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(54) Title: ENDO BETA-1,4-GLUCANASE FROM ASPERGILLUS

(57) Abstract

A glucanase enzyme is described. In addition, there is described a nucleotide sequence coding for the same and a promoter for controlling the expression of the same.

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ENDO BETA-1,4-GLUCANASE FROM ASPERGILLUS

The present invention relates to an enzyme. In addition, the present invention relates to a nucleotide sequence coding for the enzyme. Also, the present invention relates to a promoter, wherein the promoter can be used to control the expression of the nucleotide sequence coding for the enzyme.

In particular, the enzyme of the present invention is a glucanase enzyme - i.e. an enzyme that can degrade β-1,4-glucosidic bonds.

It is known that it is desirable to direct expression of a gene of interest ("GOI") in certain tissues of an organism - such as a filamentous fungus (such as *Aspergillus niger*) or even a plant crop. The resultant protein or enzyme may be useful for the organism itself. For example, it may be desirable to produce crop protein products with an optimised amino acid composition and so increase the nutritive value of a crop. For example, the crop may be made more useful as a feed. In the alternative, it may be desirable to isolate the resultant protein or enzyme and then use the protein or enzyme to prepare, for example, food compositions. In this regard, the resultant protein or enzyme can be a component of the food composition or it can be used to prepare food compositions, including altering the characteristics or appearance of food compositions.

It may even be desirable to use the organism, such as a filamentous fungus or a crop plant, to express non-plant genes, such as for the same purposes.

Also, it may be desirable to use an organism, such as a filamentous fungus or a crop plant, to express mammalian genes. Examples of the latter products include interferons, insulin, blood factors and plasminogen activators.

It is also desirable to use micro-organisms, such as filamentous fungi, to prepare products from GOIs by use of promoters that are active in the micro-organisms.

Fruit and vegetable cell walls largely consist of polysaccharide, the major components being pectin, cellulose and xyloglucan, R.R. Selvendran and J.A. Robertson, IFR Report 1989. Numerous cell wall models have been proposed which attempt to incorporate the essential properties of strength and flexibility (P. Albersheim, Sci. Am. 232, 81-95, 1975;, P. Albersheim, Plant Biochem. 3rd Edition (Bonner and Varner), Ac. Press, 1976; T. Hayashi, Ann. Rev. Plant Physiol. & Plant Mol. Biol., 40, 139-168, 1989).

The composition of the plant cell wall is complex and variable. Polysaccharides are mainly found in the form of long chains of cellulose (the main structural component of the plant cell wall), hemicellulose (comprising various ß-xylan chains, such as xyloglucans) and pectic substances (consisting of galacturonans and rhamnogalacturonans; arabinans; and galactans and arabinogalactans).

In particular, glucans are polysaccharides made up exclusively of glucose subunits. Typical examples of glucans are starch and cellulose.

The enzymes that degrade glucans are collectively referred to as glucanases. A typical glucanase is β-1,4-endoglucanase.

β-1,4-endoglucanases have uses in many industries. For example, in the brewing industry, barley is used for production of malt, and, in the latter years, as adjunct in the brewing process. When the quality of the malt is poor, or barley has been used as an adjunct, problems with high viscosity in the wort can arise because of β-glucans from the barley. In this regard, barley contains large quantities of mixed β-1.3/1,4- glucans of very high molecular weight. When dissolved, these glucans produce high viscosity solutions, which can cause troubles in some applications. For example, the high viscosity reduces the filterability of the wort and can lead to unacceptable long filtration times. To avoid these problems β-glucanase has been traditionally added to wort to avoid such problems - i.e. the problem with glucans can be avoided by addition of enzymes, in particular, glucanases, which degrade the polymers.

Further information on these problems may be found in the Grindsted brochure called "Glucanase GV", the reviews by Dr. C.W. Bamforth (Brewers Digest June 1982 pages 22-28; and Brewers' Guardian September 1985 pages 21-26), and the paper by T.Godfrey (Industrial Enzymology The Application of Enzymes in Industry Chapter 4.5 pages 221-259).

In the feed industry barley can be used for chicken feed because it is cheap, but again the β-glucan can give problems for the digestion of the chicken. By addition of β-glucanase to the feed the digestibility of the feed can be increased. In addition, the faeces of chickens feeding on feed containing barley is sticky making it difficult to remove and results in dirty eggs.

WO 93/2019 discusses endo-ß-1,4-glucanases (EC no. 3.2.1.4). According to WO 93/2019, these glucanases are a group of hydrolases which catalyse endo hydrolysis of 1,4-ß-D-glycosidic linkages in cellulose, lichenin, cereal ß-D-glucans and other plant material containing cellulosic parts. Endo-1,4-ß-D-glucan 4-glucano hydrolase is sometimes called endo-ß-1,4-glucanase.

The endo-ß-1.4-glucanase of WO 93/2019 exhibits a pH-optimum of 2.0 to 4.0, an isoelectric point of 2.0 to 3.5, a molecular weight of between 30,000 and 50,000, and a temperature optimum between 30 and 70°C.

Further teachings on glucans may be found in WO 93/17101, in particular xyloglucans. According to WO 93/17101 the xyloglucans are 1,4-ß-glucans that have been extensively substituted with α-1,6-xylosyl side chains, some of which are 1,2-ß-galactosylated. They are found in large amounts in the primary cell walls of dicots but also in certain seeds, where they serve different roles. Primary cell wall xyloglucan is fucosylated. Xyloglucan is tightly hydrogen bonded to cellulose microfibrils and requires concentrated alkali or strong swelling agents to release it. Xyloglucan is thought to form cross-bridges between cellulose microfibrils, the cellulose/xyloglucan network forming the major load-bearing/elastic network of the wall. DCB mutated suspension culture cells (cell walls lacking cellulose) release xyloglucan into their media, suggesting that xyloglucan is

normally rightly bound to cellulose.

Hydrolysis of primary cell wall xyloglucan has been demonstrated in segments of dark grown squash hypocotyls, during IAA induced growth (K. Wakabayashi et al, Plant Physiol., 95, 1070-1076, 1991). Endohydrolysis of wall xyloglucan is thought to contribute to wall loosening which accompanies cell expansion (T. Hyashi, Ann. Rev. Plant Physiol. & Plant Mol. Biol., 40, 139-168, 1989). The average molecular weight of xyloglucan has also been shown to decrease during tomato fruit ripening and this may contribute to the tissue softening which accompanies the ripening process (D.J. Huber, J. Amer. Soc. Hort. Sci., 108(3), 405-409, 1983). Certain seeds, e.g. Nasturtium, contain up to 30% by weight of xyloglucan, stored in thickened cotyledonary cell walls, which serves as a reserve polysaccharide and is rapidly depolymerised during germination.

It would be useful to increase glucanase activity, for example to have a plant with high concentration of glucanase for use in feed, preferably using recombinant DNA techniques.

The present invention seeks to provide an enzyme having glucanase activity; preferably wherein the enzyme can be prepared in certain or specific cells or tissues, such as in just a specific cell or tissue, of an organism, typically a filamentous fungus, preferably of the genus *Aspergillus*, such as *Aspergillus niger*, or even a plant.

Also, the present invention seeks to provide a GOI coding for the enzyme that can be expressed preferably in specific cells or tissues, such as in certain or specific cells or tissues, of an organism, typically a filamentous fungus, preferably of the genus *Aspergillus*, such as *Aspergillus niger*, or even a plant.

In addition, the present invention seeks to provide a promoter that is capable of directing expression of a GOI, such as a nucleotide sequence coding for the enzyme according to the present invention, preferably in certain specific cells or tissues, such as in just a specific cell or tissue, of an organism, typically a filamentous fungus, preferably of the

genus *Aspergillus*, such as *Aspergillus niger*, or even a plant. Preferably, the promoter is used in *Aspergillus* wherein the product encoded by the GOI is excreted from the host organism into the surrounding medium.

Furthermore, the present invention seeks to provide constructs, vectors, plasmids, cells, tissues, organs and organisms comprising the GOI and/or the promoter, and methods of expressing the same, preferably in specific cells or tissues, such as expression in just a specific cell or tissue, of an organism, typically a filamentous fungus, preferably of the genus *Aspergillus*, or even a plant.

According to a first aspect of the present invention there is provided an enzyme obtainable from *Aspergillus*, wherein the enzyme has the following characteristics: a MW of 24,235 D ± 50 D; a pI value of about 4; glucanase activity; and wherein the glucanase activity is endo β-1,4-glucanase activity.

According to a second aspect of the present invention there is provided an enzyme having the sequence shown as SEQ. I.D. No. 1 or a variant, homologue or fragment thereof.

According to a third aspect of the present invention there is provided an enzyme coded by the nucleotide sequence shown as SEQ. I.D. No. 2 or a variant, homologue or fragment thereof or a sequence complementary thereto.

According to a fourth aspect of the present invention there is provided a nucleotide sequence coding for the enzyme according to the present invention.

According to a fifth aspect of the present invention there is provided a nucleotide sequence having the sequence shown as SEQ. I.D. No. 2 or a variant, homologue or fragment thereof or a sequence complementary thereto.

According to a sixth aspect of the present invention there is provided a promoter having the sequence shown as SEQ. I.D. No. 3 or a variant, homologue or fragment thereof or a sequence complementary thereto.

According to a seventh aspect of the present invention there is provided a terminator having the nucleotide sequence shown as SEQ. I.D. No. 13 or a variant, homologue or fragment thereof or a sequence complementary thereto.

According to an eighth aspect of the present invention there is provided a signal sequence having the nucleotide sequence shown as SEQ. I.D. No. 14 or a variant, homologue or fragment thereof or a sequence complementary thereto.

According to a ninth aspect of the present invention there is provided a process for expressing a GOI by use of a promoter, wherein the promoter is the promoter according to the present invention.

According to a tenth aspect of the present invention there is provided the use of an enzyme according to the present invention to degrade a glucan.

According to an eleventh aspect of the present invention there is provided plasmid NCIMB 40704, or a nucleotide sequence obtainable therefrom for expressing an enzyme capable of degrading arabinoxylan or for controlling the expression thereof or for controlling the expression of another GOI.

According to a twelfth aspect of the present invention there is provided a signal sequence having the sequence shown as SEQ. I.D. No. 15 or a variant, homologue or fragment thereof.

According to a thirteenth aspect of the present invention there is provided a glucanase enzyme having the ability to degrade β-1,4-glucosidic bonds, which is immunologically reactive with an antibody raised against a purified glucanase enzyme having the sequence shown as SEQ. I.D. No. 1.

According to a fourteenth aspect of the present invention there is provided a promoter that is inducible by glucose.

According to a fifteenth aspect of the present invention there is provided the use of glucose to induce a promoter.

Other aspects of the present invention include constructs, vectors, plasmids, cells, tissues, organs and transgenic organisms comprising the aforementioned aspects of the present invention.

Other aspects of the present invention include methods of expressing or allowing expression or transforming any one of the nucleotide sequence, the construct, the plasmid, the vector, the cell, the tissue, the organ or the organism, as well as the products thereof.

Additional aspects of the present invention include uses of the promoter for expressing GOIs in culture media such as a broth or in a transgenic organism.

Further aspects of the present invention include uses of the enzyme for preparing or treating foodstuffs, including animal feed.

In the following text, the enzyme of the present invention is sometimes referred to as Egla enzyme and the coding sequence therefor is sometimes referred to as the Egla gene. In addition, the promoter of the present invention is sometimes referred to as Egla promoter.

Preferably the enzyme is coded by the nucleotide sequence shown as SEQ. I.D. No. 2 or a variant, homologue or fragment thereof or a sequence complementary thereto.

Preferably the nucleotide sequence has the sequence shown as SEQ. I.D. No. 2 or a variant, homologue or fragment thereof or a sequence complementary thereto.

Preferably the nucleotide sequence is operatively linked to a promoter.

Preferably the promoter is the promoter having the sequence shown as SEQ. I.D. No. 3 or a variant, homologue or fragment thereof or a sequence complementary thereto.

Preferably the promoter of the present invention is operatively linked to a GOI.

Preferably the GOI comprises a nucleotide sequence according to the present invention.

In one preferred embodiment, the transgenic organism is a fungus. For example the organism can be a yeast, which would then be useful in for example the brewing industry.

Preferably the transgenic organism is a filamentous fungus, more preferably of the genus *Aspergillus*.

In another preferred embodiment the transgenic organism is a plant.

In another preferred embodiment the transgenic organism is a yeast. In this regard, yeast have been widely used as a vehicle for heterologous gene expression. The species *Saccharomyces cerevisiae* has a long history of industrial use, including use for heterologous gene expression. Expression of heterologous genes in *Saccharomyces cerevisiae* has been reviewed by Goodey *et al* (1987, Yeast Biotechnology, D R Berry *et al*, eds, pp 401-429, Allen and Unwin, London) and by King *et al* (1989, Molecular and Cell Biology of Yeasts, E F Walton and G T Yarronton, eds, pp 107-133, Blackie, Glasgow).

For several reasons *Saccharomyces cerevisiae* is well suited for heterologous gene expression. First, it is non-pathogenic to humans and incapable of producing certain endotoxins. Second, it has a long history of safe use following centuries of commercial exploitation for various purposes. This has led to wide public acceptability. Third, the extensive commercial use and research devoted to the organism has resulted in a wealth of knowledge about the genetics and physiology as well as large-scale fermentation characteristics of *Saccharomyces cerevisiae*.

An additional advantage is that yeasts are capable of post-translational modifications of proteins and thereby have the potential for glycosylation and/or secretion of heterologous gene products into the growth medium. A review of the principles of heterologous gene expression in *Saccharomyces cerevisiae* and secretion of gene products is given by E Hinchcliffe E Kenny (1993, "Yeast as a vehicle for the expression of heterologous genes", Yeasts, Vol 5, Anthony H Rose and J Stuart Harrison, eds, 2nd edition, Academic Press Ltd.).

The glycosylation of enzymes expressed in yeast is known to increase heat stability of the enzyme. Enhancing the heat stability of the glucanase according to the present invention will make this enzyme suitable for use in the brewing industry and for use in the preparation of animal feed, i.e. chicken feed.

Yeasts are known to secrete very few proteins into the culture medium. This makes yeast a very attractive host for expression of heterologous genes, since secretable gene products can easily be recovered and purified.

Several types of yeast vectors are available, including integrative vectors, which require recombination with the host genome for their maintenance, and autonomously replicating plasmid vectors.

In order to prepare the transgenic *Saccharomyces*, expression constructs are prepared by inserting a GOI (such as an amylase or SEQ. ID No 2) into a construct designed for expression in yeast. Several types of constructs used for heterologous expression have been developed. The constructs contain a promoter active in yeast fused to the GOI, usually a promoter of yeast origin, such as the GAL1 promoter, is used. The GOI can be fused to a signal sequence which directs the protein encoded by the GOI to be secreted. Usually a signal sequence of yeast origin, such as the sequence encoding the SUC2 signal peptide, is used. A terminator active in yeast ends the expression system.

Heterologous expression in yeast has been reported for several genes. The gene products can be glycosylated which is advantageous for some enzymes intended for specific

application where heat tolerance is desirable. The proteins can be deposited intracellularly if the GOI is not fused to a signal sequence, or they can be secreted extracelluarly if the GOI is equipped with a signal sequence.

For the transformation of yeast several transformation protocols have been developed.

For example, the transgenic *Saccharomyces* according to the present invention can be prepared by following the teachings of Hinnen *et al* (1978, Proceedings of the National Academy of Sciences of the USA 75, 1929) Beggs, J D (1978, Nature, London, 275, 104); and Ito, H *et al* (1983, J Bacteriology 153, 163-168).

The transformed yeast cells are selected using various selective markers. Among the markers used for transformation are a number of auxotrophic markers such as LEU2, HIS4 and TRP1, and dominant antibiotic resistance markers such as aminoglycoside antibiotic markers, eg G418.

Highly preferred embodiments of each of the aspects of the present invention do not include any one of the native enzyme, the native promoter or the native nucleotide sequence in its natural environment.

Preferably, in any one of the plasmid, the vector such as an expression vector or a transformation vector, the cell, the tissue, the organ, the organism or the transgenic organism, the promoter is present in combination with at least one GOI.

Preferably the promoter and the GOI are stably incorporated within the transgenic organism's genome.

Preferably the transgenic organism is a filamentous fungus, preferably of the genus *Aspergillus*, more preferably *Aspergillus niger*. Alternatively, the transgenic organism can be a yeast. The transgenic organism can even be a plant, such as a monocot or dicot plant.

A highly preferred embodiment is an enzyme obtainable from *Aspergillus*, wherein the enzyme has the following characteristics: a MW of 24,235 D ± 50 D; a pI value of about 4; glucanase activity; and wherein the glucanase activity is endo β-1,4-glucanase activity; wherein the enzyme has the sequence shown as SEQ. I.D. No. 1 or a variant, homologue or fragment thereof.

Another highly preferred embodiment is an enzyme obtainable from *Aspergillus*, wherein the enzyme has the following characteristics: a MW of 24,235 D ± 50 D; a pI value of about 4; glucanase activity; and wherein the glucanase activity is endo β-1,4-glucanase activity; wherein the enzyme is coded by the nucleotide sequence shown as SEQ. I.D. No. 2 or a variant, homologue or fragment thereof or a sequence complementary thereto.

The advantages of the present invention are that it provides a means for preparing a glucanase enzyme and the nucleotide sequence coding for the same. In addition, it provides a promoter that can control the expression of that, or another, nucleotide sequence.

Other advantages of the present invention are that the enzyme can be used to prepare useful feeds containing cereals, such as barley, maize, rice etc.

The present invention therefore provides an enzyme having glucanase activity wherein the enzyme can be prepared in certain or specific cells or tissues, such as in just a specific cell or tissue, of an organism, typically a filamentous fungus, preferably of the genus *Aspergillus*, such as *Aspergillus niger*. The enzyme may even be prepared in a plant.

Also, the present invention provides a GOI coding for the enzyme that can be expressed preferably in specific cells or tissues, such as in certain or specific cells or tissues, of an organism, typically a filamentous fungus, preferably of the genus *Aspergillus*, such as *Aspergillus niger*. The GOI may even be expressed in a plant.

In addition, the present invention provides a promoter that is capable of directing expression of a GOI, such as a nucleotide sequence coding for the enzyme according to the present invention, preferably in certain specific cells or tissues, such as in just a specific cell or tissue, of an organism, typically a filamentous fungus, preferably of the genus *Aspergillus*, such as *Aspergillus niger*, or even a plant. Preferably, the promoter is used in *Aspergillus* wherein the product encoded by the GOI is excreted from the host organism into the surrounding medium. The promoter may even be tailored (if necessary) to express a GOI in a plant.

The present invention also provides constructs, vectors, plasmids, cells, tissues, organs and organisms comprising the GOI and/or the promoter, and methods of expressing the same, preferably in specific cells or tissues, such as expression in just a specific cell or tissue, of an organism, typically a filamentous fungus, preferably of the genus *Aspergillus*, or even a plant.

The terms "variant", "homologue" or "fragment" in relation to the enzyme include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acid from or to the sequence providing the resultant amino acid sequence has glucanase activity, preferably having at least the same activity of the enzyme shown in the sequence listings (SEQ I.D. No. 1 or 12). In particular, the term "homologue" covers homology with respect to structure and/or function providing the resultant enzyme has glucanase activity. With respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to SEQ ID NO. 1 shown in the attached sequence listings. More preferably there is at least 95%, more preferably at least 98%, homology to SEQ ID NO. 1 shown in the attached sequence listings.

The terms "variant", "homologue" or "fragment" in relation to the nucleotide sequence coding for the enzyme include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence providing the resultant nucleotide sequence codes for an enzyme having glucanase activity, preferably having at least the same activity of the enzyme shown in

the sequence listings (SEQ I.D. No. 2 or 12). In particular, the term "homologue" covers homology with respect to structure and/or function providing the resultant nucleotide sequence codes for an enzyme having glucanase activity. With respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to SEQ ID NO. 2 shown in the attached sequence listings. More preferably there is at least 95%, more preferably at least 98%, homology to SEQ ID NO. 2 shown in the attached sequence listings.

The terms "variant", "homologue" or "fragment" in relation to the promoter include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence providing the resultant nucleotide sequence has the ability to act as a promoter in an expression system - such as the transformed cell or the transgenic organism according to the present invention. In particular, the term "homologue" covers homology with respect to structure and/or function providing the resultant nucleotide sequence has the ability to act as a promoter. With respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to SEQ ID NO. 3 shown in the attached sequence listings. More preferably there is at least 95%, more preferably at least 98%, homology to SEQ ID NO. 3 shown in the attached sequence listings.

The terms "variant", "homologue" or "fragment" in relation to the terminator or signal nucleotide sequences include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence providing the resultant nucleotide sequence has the ability to act as a terminator or codes for an amino acid sequence that has the ability to act as a signal sequence respectively in an expression system - such as the transformed cell or the transgenic organism according to the present invention. In particular, the term "homologue" covers homology with respect to structure and/or function providing the resultant nucleotide sequence has the ability to act as or code for a terminator or signal respectively. With respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to SEQ ID NO.s 13 and 14 (respectively) shown in the attached sequence listings. More preferably there is at least 95%, more

preferably at least 98%, homology to SEQ ID NO.s 13 and 14 (respectively) shown in the attached sequence listings.

The terms "variant", "homologue" or "fragment" in relation to the signal amino acid sequence include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acid from or to the sequence providing the resultant sequence has the ability to act as a signal sequence in an expression system - such as the transformed cell or the transgenic organism according to the present invention. In particular, the term "homologue" covers homology with respect to structure and/or function providing the resultant nucleotide sequence has the ability to act as or code for a signal. With respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to SEQ ID NO 15 shown in the attached sequence listings. More preferably there is at least 95%, more preferably at least 98%, homology to SEQ ID NO 15 shown in the attached sequence listings.

The above terms are synonymous with allelic variations of the sequences.

The term "complementary" means that the present invention also covers nucleotide sequences that can hybridise to the nucleotide sequences of the coding sequence, the promoter sequence, the terminator sequence or the signal sequence respectively.

The term "nucleotide" in relation to the present invention includes genomic DNA, CDNA, synthetic DNA, and RNA. Preferably it means DNA, more preferably cDNA for the coding sequence of the present invention since the genomic coding sequence has two introns and their removal would allow expression in bacteria.

The term "construct" - which is synonymous with terms such as "conjugate", "cassette" and "hybrid" - includes a GOI directly or indirectly attached to a promoter. An example of an indirect attachment is the provision of a suitable spacer group such as an intron sequence, such as the *Sh1*-intron or the ADH intron, intermediate the promoter and the GOI. The same is true for the term "fused" in relation to the present invention which

includes direct or indirect attachment. In each case, it is highly preferred that the terms do not cover the natural combination of the gene coding for the enzyme ordinarily associated with the wild type gene promoter and when they are both in their natural environment. A highly preferred embodiment is the or a GOI being operatively linked to a or the promoter.

The construct may even contain or express a marker which allows for the selection of the genetic construct in, for example, a filamentous fungus, preferably of the genus *Aspergillus*, such as *Aspergillus niger*, or plants, preferably cereals, such as maize, rice, barley etc., into which it has been transferred. Various markers exist which may be used, such as for example those encoding mannose-6-phosphate isomerase (especially for plants) or those markers that provide for antibiotic resistance - e.g. resistance to G418, hygromycin, bleomycin, kanamycin and gentamycin.

The term "vector" includes expression vectors and transformation vectors.

The term "expression vector" means a construct capable of *in vivo* or *in vitro* expression.

The term "transformation vector" means a construct capable of being transferred from one species to another - such as from an *E.coli* plasmid to a filamentous fungus, preferably of the genus *Aspergillus*. It may even be a construct capable of being transferred from an *E.coli* plasmid to an *Agrobacterium* to a plant.

The term "tissue" includes tissue *per se* and organ.

The term "organism" in relation to the present invention includes any organism that could comprise the promoter according to the present invention and/or the nucleotide sequence coding for the enzyme according to the present invention and/or products obtained therefrom, wherein the promoter can allow expression of a GOI and/or wherein the nucleotide sequence according to the present invention can be expressed when present in the organism.

Preferably the organism is a filamentous fungus, preferably of the genus *Aspergillus*, more preferably *Aspergillus niger*.

The term "transgenic organism" in relation to the present invention includes any organism that comprises the promoter according to the present invention and/or the nucleotide sequence coding for the enzyme according to the present invention and/or products obtained therefrom, wherein the promoter can allow expression of a GOI and/or wherein the nucleotide sequence according to the present invention can be expressed within the organism. Preferably the promoter and/or the nucleotide sequence is (are) incorporated in the genome of the organism. Preferably the transgenic organism is a filamentous fungus, preferably of the genus *Aspergillus*, more preferably *Aspergillus niger*.

Therefore, the transgenic organism of the present invention includes an organism comprising any one of, or combinations of, the promoter according to the present invention, the nucleotide sequence coding for the enzyme according to the present invention, constructs according to the present invention, vectors according to the present invention, plasmids according to the present invention, cells according to the present invention, tissues according to the present invention or the products thereof. For example the transgenic organism can comprise a GOI, preferably an exogenous nucleotide sequence, under the control of the promoter according to the present invention. The transgenic organism can also comprise the nucleotide sequence coding for the enzyme of the present invention under the control of a promoter, which may be the promoter according to the present invention.

In a highly preferred embodiment, the transgenic organism does not comprise the combination of the promoter according to the present invention and the nucleotide sequence coding for the enzyme according to the present invention, wherein both the promoter and the nucleotide sequence are native to that organism and are in their natural environment. Thus, in these highly preferred embodiments, the present invention does not cover the native nucleotide coding sequence according to the present invention in its natural environment when it is under the control of its native promoter which is also in its natural environment. In addition, in this highly preferred embodiment, the present

invention does not cover the native enzyme according to the present invention when it is in its natural environment and when it has been expressed by its native nucleotide coding sequence which is also in its natural environment and when that nucleotide sequence is under the control of its native promoter which is also in its natural environment.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site in the Jacob-Mond theory of gene expression.

In one aspect, the promoter of the present invention is capable of expressing a GOI, which can be the nucleotide sequence coding for the enzyme of the present invention.

In another aspect, the nucleotide sequence according to the present invention is under the control of a promoter that allows expression of the nucleotide sequence. In this regard, the promoter need not necessarily be the same promoter as that of the present invention. In this aspect, the promoter may be a cell or tissue specific promoter. If, for example, the organism is a plant then the promoter can be one that affects expression of the nucleotide sequence in any one or more of stem, sprout, root and leaf tissues.

By way of example, the promoter for the nucleotide sequence of the present invention can be the α-Amy 1 promoter (otherwise known as the Amy 1 promoter, the Amy 637 promoter or the α-Amy 637 promoter) as described in our co-pending UK patent application No. 9421292.5 filed 21 October 1994. That promoter comprises the sequence shown in Figure 1.

Alternatively, the promoter for the nucleotide sequence of the present invention can be the α-Amy 3 promoter (otherwise known as the Amy 3 promoter, the Amy 351 promoter or the α-Amy 351 promoter) as described in our co-pending UK patent application No. 9421286.7 filed 21 October 1994. That promoter comprises the sequence shown in Figure 2.

Preferably, the promoter is the promoter of the present invention.

In addition to the nucleotide sequences described above, the promoters, particularly that of the present invention, could additionally include features to ensure or to increase expression in a suitable host. For example, the features can be conserved regions such as a Pribnow Box or a TATA box. The promoters may even contain other sequences to affect (such as to maintain, enhance, decrease) the levels of expression of the GOI. For example, suitable other sequences include the *Sh1*-intron or an ADH intron. Other sequences include inducible elements - such as temperature, chemical, light or stress inducible elements. Also, suitable elements to enhance transcription or translation may be present. An example of the latter element is the TMV 5' signal sequence (see Sleat Gene 217 [1987] 217-225; and Dawson Plant Mol. Biol. 23 [1993] 97).

In addition the present invention also encompasses combinations of promoters and/or nucleotide sequences coding for proteins or enzymes and/or elements. For example, the present invention encompasses the combination of a promoter according to the present invention operatively linked to a GOI, which could be a nucleotide sequence according to the present invention, and another promoter such as a tissue specific promoter operatively linked to the same or a different GOI.

The present invention also encompasses the use of promoters to express a nucleotide sequence coding for the enzyme according to the present invention, wherein a part of the promoter is inactivated but wherein the promoter can still function as a promoter. Partial inactivation of a promoter in some instances is advantageous.

In particular, with the Amy 351 promoter mentioned earlier it is possible to inactivate a part of it so that the partially inactivated promoter expresses GOIs in a more specific manner such as in just one specific tissue type or organ.

The term "inactivated" means partial inactivation in the sense that the expression pattern of the promoter is modified but wherein the partially inactivated promoter still functions as a promoter. However, as mentioned above, the modified promoter is capable of expressing a GOI in at least one (but not all) specific tissue of the original promoter. One such promoter is the Amy 351 promoter described above.

Examples of partial inactivation include altering the folding pattern of the promoter sequence, or binding species to parts of the nucleotide sequence, so that a part of the nucleotide sequence is not recognised by, for example, RNA polymerase. Another, and preferable, way of partially inactivating the promoter is to truncate it to form fragments thereof. Another way would be to mutate at least a part of the sequence so that the RNA polymerase can not bind to that part or another part.

Another modification is to mutate the binding sites for regulatory proteins for example the CreA protein known from filamentous fungi to exert carbon catabolite repression, and thus abolish the catabolite repression of the native promoter.

The term "GOI" with reference to the present invention means any gene of interest. A GOI can be any nucleotide that is either foreign or natural to the organism (e.g. filamentous fungus, preferably of the genus *Aspergillus*, or a plant) in question. Typical examples of a GOI include genes encoding for proteins and enzymes that modify metabolic and catabolic processes. The GOI may code for an agent for introducing or increasing pathogen resistance. The GOI may even be an antisense construct for modifying the expression of natural transcripts present in the relevant tissues. The GOI may even code for a non-natural protein of a filamentous fungus, preferably of the genus *Aspergillus*, or a compound that is of benefit to animals or humans.

For example, the GOI could code for a pharmaceutically active protein or enzyme such as any one of the therapeutic compounds insulin, interferon, human serum albumin, human growth factor and blood clotting factors. In this regard, the transformed cell or organism could prepare acceptable quantities of the desired compound which would be easily retrievable from, the cell or organism. The GOI may even be a protein giving nutritional value to a food or crop. Typical examples include plant proteins that can inhibit the formation of anti-nutritive factors and plant proteins that have a more desirable amino acid composition (e.g. a higher lysine content than a non-transgenic plant). The GOI may even code for an enzyme that can be used in food processing such as chymosin, thaumatin and α-galactosidase. The GOI can be a gene encoding for any one of a pest toxin, an antisense transcript such as that for patatin or α-amylase, ADP-glucose

pyrophosphorylase (e.g. see EP-A-0455316), a protease antisense or a glucanase.

The GOI can be the nucleotide sequence coding for the α-amylase enzyme which is the subject of our co-pending UK patent application 9413439.2 filed on 4 July 1994, the sequence of which is shown in Figure 3. The GOI can be the nucleotide sequence coding for the α-amylase enzyme which is the subject of our co-pending UK patent application 9421290.9 filed on 21 October 1994, the sequence of which is shown in Figure 4. The GOI can be any of the nucleotide sequences coding for the ADP-glucose pyrophosphorylase enzymes which are the subject of our co-pending PCT patent application PCT/EP94/01082 filed 7 April 1994, the sequences of which are shown in Figures 5 and 6. The GOI can be any of the nucleotide sequences coding for the α-glucan lyase enzyme which are described in our co-pending PCT patent application PCT/EP94/03397 filed 15 October 1994, the sequences of which are shown in Figures 7-10.

In one preferred embodiment, the GOI is a nucleotide sequence coding for the enzyme according to the present invention.

As mentioned above, a preferred host organism is of the genus *Aspergillus*, such as *Aspergillus niger*.

The transgenic *Aspergillus* according to the present invention can be prepared by following the teachings of Rambosek,J. and Leach,J. 1987 (Recombinant DNA in filamentous fungi: Progress and Prospects. CRC Crit. Rev. Biotechnol. 6:357-393), Davis R.W. 1994 (Heterologous gene expression and protein secretion in *Aspergillus*. In: Martinelli S.D., Kinghorn J.R.(Editors) *Aspergillus*: 50 years on. Progress in industrial microbiology vol 29. Elsevier Amsterdam 1994. pp 525-560), Ballance,D.J. 1991 (Transformation systems for Filamentous Fungi and an Overview of Fungal Gene structure. In :Leong,S.A., Berka R.M. (Editors) Molecular Industrial Mycology. Systems and Applications for Filamentous Fungi. Marcel Dekker Inc. New York 1991. pp 1-29) and Turner G. 1994 (Vectors for genetic manipulation. In: Martinelli S.D., Kinghorn J.R.(Editors) *Aspergillus*: 50 years on. Progress in industrial microbiology vol 29.

Elsevier Amsterdam 1994, pp. 641-666). However, the following commentary provides a summary of those teachings for producing transgenic *Aspergillus* according to the present invention.

Filamentous fungi have during almost a century been widely used in industry for production of organic compounds and enzymes. Traditional japanese koji and soy fermentations have used *Aspergillus sp.* for hundreds of years. In this century *Aspergillus niger* has been used for production of organic acids particular citric acid and for production of various enzymes for use in industry.

There are two major reasons for that filamentous fungi have been so widely used in industry. First filamentous fungi can produce high amounts of extracellular products, for example enzymes and organic compounds such as antibiotics or organic acids. Second filamentous fungi can grow on low cost substrates such as grains, bran, beet pulp etc. The same reasons have made filamentous fungi attractive organisms as hosts for heterologous expression according to the present invention.

In order to prepare the transgenic *Aspergillus*, expression constructs are prepared by inserting a GOI (such as an amylase or SEQ. I.D. No. 2) into a construct designed for expression in filamentous fungi.

Several types of constructs used for heterologous expression have been developed. The constructs contain the promoter according to the present invention (or if desired another promoter if the GOI codes for the enzyme according to the present invention) which is active in fungi. Examples of promoters other than that of the present invention include a fungal promoter for a highly expressed extracellulary enzyme, such as the glucoamylase promoter or the α-amylase promoter. The GOI can be fused to a signal sequence (such as that of the present invention or another suitable sequence) which directs the protein encoded by the GOI to be secreted. Usually a signal sequence of fungal origin is used, such as that of the present invention. A terminator active in fungi ends the expression system, such as that of the present invention.

Another type of expression system has been developed in fungi where the GOI is fused to a smaller or a larger part of a fungal gene encoding a stable protein. This can stabilize the protein encoded by the GOI. In such a system a cleavage site, recognized by a specific protease, can be introduced between the fungal protein and the protein encoded by the GOI, so the produced fusion protein can be cleaved at this position by the specific protease thus liberating the protein encoded by the GOI ("POI"). By way of example, one can introduce a site which is recognized by a KEX-2 like peptidase found in at least some *Aspergilli*. Such a fusion leads to cleavage *in vivo* resulting in protection of the POI and production of POI and not a larger fusion protein.

Heterologous expression in *Aspergillus* has been reported for several genes coding for bacterial, fungal, vertebrate and plant proteins. The proteins can be deposited intracellularly if the GOI is not fused to a signal sequence. Such proteins will accumulate in the cytoplasm and will usually not be glycosylated which can be an advantage for some bacterial proteins. If the GOI is equipped with a signal sequence the protein will accumulate extracellulary.

With regard to product stability and host strain modifications, some heterologous proteins are not very stable when they are secreted into the culture fluid of fungi. Most fungi produce several extracellular proteases which degrade heterologous proteins. To avoid this problem special fungal strains with reduced protease production have been used as host for heterologous production.

For the transformation of filamentous fungi, several transformation protocols have been developed for many filamentous fungi (Ballance 1991, *ibid*). Many of them are based on preparation of protoplasts and introduction of DNA into the protoplasts using PEG and Ca^{2+} ions. The transformed protoplasts then regenerate and the transformed fungi are selected using various selective markers. Among the markers used for transformation are a number of auxotrophic markers such as *argB*, *trpC*, *niaD* and *pyrG*, antibiotic resistance markers such as benomyl resistance, hygromycin resistance and phleomycin resistance. A very common used transformation marker is the *amdS* gene of *A. nidulans* which in high copy number allows the fungus to grow with acrylamide as the sole

nitrogen source.

Even though the enzyme, the nucleotide sequence coding for same and the promoter of the present invention are not disclosed in EP-B-0470145 and CA-A-2006454, those two documents do provide some useful background commentary on the types of techniques that may be employed to put the present invention into practice. Some of these background teachings are now included in the following commentary.

The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material. Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system.

A review of the general techniques may be found in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27).

Thus, in one aspect, the present invention relates to a vector system which carries a promoter or nucleotide sequence or construct according to the present invention and which is capable of introducing the promoter or nucleotide sequence or construct into the genome of an organism, such as a plant.

The vector system may comprise one vector, but it can comprise two vectors. In the case of two vectors, the vector system is normally referred to as a binary vector system. Binary vector systems are described in further detail in Gynheung An et al. (1980). Binary Vectors, *Plant Molecular Biology Manual A3*, 1-19.

One extensively employed system for transformation of plant cells with a given promoter or nucleotide sequence or construct is based on the use of a Ti plasmid from *Agrobacterium tumefaciens* or a Ri plasmid from *Agrobacterium rhizogenes* An et al. (1986), *Plant Physiol.* 81, 301-305 and Butcher D.N. et al. (1980), *Tissue Culture*

Methods for Plant Pathologists, eds.: D.S. Ingrams and J.P. Helgeson, 203-208.

Several different Ti and Ri plasmids have been constructed which are suitable for the construction of the plant or plant cell constructs described above. A non-limiting example of such a Ti plasmid is pGV3850.

The promoter or nucleotide sequence or construct of the present invention should preferably be inserted into the Ti-plasmid between the terminal sequences of the T-DNA or adjacent a T-DNA sequence so as to avoid disruption of the sequences immediately surrounding the T-DNA borders, as at least one of these regions appear to be essential for insertion of modified T-DNA into the plant genome.

As will be understood from the above explanation, if the organism is a plant, then the vector system of the present invention is preferably one which contains the sequences necessary to infect the plant (e.g. the *vir* region) and at least one border part of a T-DNA sequence, the border part being located on the same vector as the genetic construct.

Furthermore, the vector system is preferably an *Agrobacterium tumefaciens* Ti-plasmid or an *Agrobacterium rhizogenes* Ri-plasmid or a derivative thereof, as these plasmids are well-known and widely employed in the construction of transgenic plants, many vector systems exist which are based on these plasmids or derivatives thereof.

In the construction of a transgenic plant the promoter or nucleotide sequence or construct of the present invention may be first constructed in a microorganism in which the vector can replicate and which is easy to manipulate before insertion into the plant. An example of a useful microorganism is *E. coli*, but other microorganisms having the above properties may be used. When a vector of a vector system as defined above has been constructed in *E. coli*, it is transferred, if necessary, into a suitable *Agrobacterium* strain, e.g. *Agrobacterium tumefaciens*. The Ti-plasmid harbouring the promoter or nucleotide sequence or construct of the invention is thus preferably transferred into a suitable *Agrobacterium* strain, e.g. *A. tumefaciens*, so as to obtain an *Agrobacterium* cell harbouring the promoter or nucleotide sequence or construct of the invention, which

DNA is subsequently transferred into the plant cell to be modified.

As reported in CA-A-2006454, a large amount of cloning vectors are available which contain a replication system in *E. coli* and a marker which allows a selection of the transformed cells. The vectors contain for example pBR 322, pUC series, M13 mp series, pACYC 184 etc. In such a way, the nucleotide or construct or promoter of the present invention can be introduced into a suitable restriction position in the vector. The contained plasmid is used for the transformation in *E.coli*. The *E.coli* cells are cultivated in a suitable nutrient medium and then harvested and lysed. The plasmid is then recovered. As a method of analysis there is generally used sequence analysis, restriction analysis, electrophoresis and further biochemical-molecular biological methods. After each manipulation, the used DNA sequence can be restricted and connected with the-next DNA sequence. Each sequence can be cloned in the same or different plasmid. After each introduction method of the desired promoter or construct or nucleotide sequence according to the present invention in the plants the presence and/or insertion of further DNA sequences may be necessary. If, for example, for the transformation the Ti- or Ri-plasmid of the plant cells is used, at least the right boundary and often however the right and the left boundary of the Ti- and Ri-plasmid T-DNA, as flanking areas of the introduced genes, can be connected. The use of T-DNA for the transformation of plant cells has been intensively studied and is described in EP-A-120516; Hoekema, in: The Binary Plant Vector System Offset-drukkerij Kanters B.B., Alblasserdam, 1985, Chapter V; Fraley, et al., Crit. Rev. Plant Sci., 4:1-46; and An et al., EMBO J. (1985) 4:277-284.

Direct infection of plant tissues by *Agrobacterium* is a simple technique which has been widely employed and which is described in Butcher D.N. et al. (1980), *Tissue Culture Methods for Plant Pathologists*, eds.: D.S. Ingrams and J.P. Helgeson, 203-208. For further teachings on this topic see Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27). With this technique, infection of a plant may be done on a certain part or tissue of the plant, i.e. on a part of a leaf, a root, a stem or another part of the plant.

Typically, with direct infection of plant tissues by *Agrobacterium* carrying the promoter and/or the GOI, a plant to be infected is wounded, e.g. by cutting the plant with a razor or puncturing the plant with a needle or rubbing the plant with an abrasive. The wound is then inoculated with the *Agrobacterium*. The inoculated plant or plant part is then grown on a suitable culture medium and allowed to develop into mature plants.

When plant cells are constructed, these cells may be grown and maintained in accordance with well-known tissue culturing methods such as by culturing the cells in a suitable culture medium supplied with the necessary growth factors such as amino acids, plant hormones, vitamins, etc. Regeneration of the transformed cells into genetically modified plants may be accomplished using known methods for the regeneration of plants from cell or tissue cultures, for example by selecting transformed shoots using an antibiotic and by subculturing the shoots on a medium containing the appropriate nutrients, plant hormones, etc.

Further teachings on plant transformation may be found in EP-A-0449375.

In summation, the present invention provides a glucanase enzyme and the nucleotide sequence coding for the same. In addition, it provides a promoter that can control the expression of that, or another, nucleotide sequence. In addition it includes terminator and signal sequences for the same.

The following sample was deposited in accordance with the Budapest Treaty at the recognised depositary The National Collections of Industrial and Marine Bacteria Limited (NCIMB) at 23 St. Machar Drive, Aberdeen, Scotland, United Kingdom, AB2 1RY on 16 January 1995:

> *E.coli* containing plasmid pEGLA-3 {i.e. *E.coli* DH5α-pEGLA-3}. The deposit number is NCIMB 40704.

The present invention will now be described by way of example.

In the following Examples reference is made to the accompanying Figures in which

Figures 1-10 are sequences of promoters and GOIs of earlier patent applications that are useful for use with the aspects of the present invention;

Figure 11 is a plasmid map of plasmid pEGLA-3;

Figure 12 is a schematic diagram of some promoter deletions;

Figure 13 is a plasmid map of pGPAMY;

Figure 14 is a graph;

Figure 15 is a plasmid map of pGP-GssAMY-Hyg;

Figure 16 is a graph; and

Figure 17 is a Western Blot.

The following Examples discuss recombinant DNA techniques. General teachings of recombinant DNA techniques may be found in Sambrook,J., Fritsch, E.F., Maniatis T. (Editors) Molecular Cloning, A laboratory manual. Second edition. Cold Spring Harbour Laboratory Press. New York 1989.

Purification of the β-glucanase

Aspergillus niger 3M43 was grown in medium containing wheat bran and beet pulp. The fermentation broth was separated from the solid part of the broth by filtration. Concentrated fermentation broth was then loaded on a 25X100mm Q-SEPHAROSE (Pharmacia) high Performance column, equilibrated with 20 mM Tris. HCl pH 7.5, and a linear gradient from 0-500 mM NaCl was performed and fractions of the eluate was collected. The β-glucanase eluted at ca 100 mM NaCl. The fractions containing

glucanase were combined and desalted using a 50x200 mm G-25 SEPHAROSE Superfine (Pharmacia). The column was then eluted with distilled water. After desalting the enzyme was concentrated using High-Trap spin columns.

Next the concentrated and desalted fractions were subjected to gel filtration on a 50x600 mm SUPERDEX 50 column. The sample was loaded and the column was eluted with 0.2 M Phosphate buffer pH 7.0 plus 0.2 M NaCl, and fractions of the eluate were collected. The fractions containing glucanase were combined and desalted and concentrated as described above.

The combined fractions were loaded on a 16X100 mm PhenylSEPHAROSE High Performance column (Pharmacia), equilibrated with 50 mM Phosphate buffer pH 6.0, containing 1.5 M $(NH_4)_2SO_4$. A gradient where the $(NH_4)_2SO_4$ concentration was varied from 1.5 - 0 M was applied and the eluate collected in fractions. The fractions containing glucanase were combined. The purity of the β-1,4-glucanase was evaluated SDS-PAGE using the Phast system gel (Pharmacia).

Characterization

The molecular weight of the purified glucanase was determined by mass spectrometry using laser desorption technology. The MW of the glucanase was found to be 24,235 D ± 50 D.

The pI value was determined by use of a Broad pI Kit (Pharmacia). The glucanase has a pI value of about 4.

After SDS-PAGE analysis, treatment PAS reagent showed that the glucanase is not glycosylated. The PAS staining was done according to the procedure of I. Van-Seuningen and M. Davril (1992) Electrophoresis 13 pp 97-99.

Amino acid sequencing of the β-glucanase

The enzyme was digested with endoproteinase Lys-C sequencing grade from Boehringer Mannheim using a modification of the method described by Stone & Williams 1993 (Stone, K.L. and Williams, K.R. (1993). Enzymatic digestion of Proteins and HPLC Peptide Isolation. In : Matsudaira P. (Editor). A practical Guide to Protein and Peptide Purification for Microsequencing. Second Edition. Academic Press, San Diego 1993. pp 45-73).

Freeze dried β-glucanase (0.4 mg) was dissolved in 50 µl of 8M urea, 0.4 M NH_4HCO_3, pH 8.4. After overlay with N_2 and addition of 5 µl of 45 mM DTT, the protein was denatured and reduced for 15 min at 50°C under N_2. After cooling to RT, 5 µl of 100 mM iodoacetamide was added for the cysteines to be derivatised for 15 min at RT in the dark under N_2. Subsequently, 90 µl of water and 5 µg of endoproteinase Lys-C in 50 µl of 50 mM Tricine and 10 mM EDTA, pH 8.0, was added and the digestion was carried out for 24h at 37°C under N_2. The resulting peptides were separated by reversed phase HPLC on a VYDAC C18 column (0.46 x 15 cm; 10 µm; The Separations Group; California) using solvent A: 0.1% TFA in water and solvent B: 0.1% TFA in acetonitrile. Selected peptides were rechromatographed on a Develosil C18 column (0.46 x 10 cm; 3µm) using the same solvent system prior to sequencing on an Applied Biosystems 476A sequencer using pulsed-liquid fast cycles.

The following peptide sequences were found:

SEQ I.D. No. 4

SEQ I.D. No. 5

SEQ I.D. No. 6

SEQ I.D. No. 7

SEQ I.D. No. 8

Isolation of a PCR clone of a fragment of the gene

PCR primers were synthesised using an Applied Biosystems DNA synthesiser model 392. In this regard, PCR primers were synthesized from two of the found peptide sequences, WEVWYGT from Seq I.D. No. 4 and WTWSGG from Seq I.D. No. 7. The primer derived from WEVWYGT (reversed) is shown as Seq I.D. No. 9 and the primer derived from WTWSGG is shown as Seq I.D. No. 10 - see below:

SEQ. I.D. No. 10
TGG ACN TGG WSN GGN GG
17 mer 256 mixture

SEQ. I.D. No. 9
CTN CCR TAC CAN ACY TCC CA
20 mer 64 mixture

PCR amplification was performed with 100 pmol of each of these primers in 100 µl reactions using the Amplitaq II kit (Perkin Elmer). The program was:

STEP	TEMP	TIME
1	94°C	2 min
2	94°C	1 min
3	55°C	2 min
4	72°C	2 min
5	72°C	5 min
6	5°C	SOAK

Steps 2-4 were repeated for 40 cycles.

The program was run on a PERKIN ELMER DNA Thermal Cycler.

A 350 bp amplified fragment was isolated and cloned into a pT7-Blue T-vector according to the manufacturer's instructions (Novagen). A fragment was isolated and sequenced. The found sequence showed that it was indeed a part of the glucanase gene.

Isolation of *A. niger* genomic DNA

1g. of frozen *A. niger* mycelium was ground in a mortar under liquid nitrogen. Following evaporation of the nitrogen cover, the ground mycelium was extracted with 15ml of an extraction buffer (100mM Tris HCl, pH 8.0, 0.50mM EDTA, 500mM NaCl, 10mM ß-mercaptoethanol) containing 1ml 20% sodium dodecyl sulphate. After incubation at 65°C for 10 min. 5ml 5M KAc, pH 5.0, was added and the mixture further incubated, after mixing, on ice for 20 mins. The mixture was then centrifuged for 20 mins. and the supernatant mixed with 0.6 vol. isopropanol to precipitate the extracted DNA. After further centrifugation for 15 mins. the DNA pellet was dissolved in 0.7 ml TE (10mM Tris, HCl pH 8.0, 1mM EDTA) and precipitated with 75 µl 3M NaAc, pH 4.8, and 500 µl isopropanol. After centrifugation the pellet was washed with 70% ETOH and dried under vacuum. The DNA was dissolved in 200 µl TE and stored at -20°C.

Construction of a library

20 µg genomic DNA was partly digested with Tsp509I, which gives ends which are compatible with EcoRI ends. The digested DNA was separated on a 1 % agarose gel and fragments of 4-10 kb was purified. A λZAPII EcoRI/CIAP kit from Stratagene was used for library construction according to the manufacturers instructions. 2 µl of the ligation (totally 5 µl) was packed with Gigapack Gold II packing extract according to the manufacturer's instructions (Stratagene). The library contained 650.000 independent clones.

Screening of the library

2 X 50.000 pfu was plated on NZY plates (5g NaCl, 2mg $MgSO_4.7H_2O$, 5g yeast extract, 10g casein hydrolysate, 15 g agar per liter) and plaquelifts were done on Hybond N sheets (Amersham). The sheets were hybridized with the PCR clone labelled with ^{32}P dCTP (Amersham) using Ready-to-go labelling kit from Pharmacia. The plaquelifts and hybridization were done in duplicate and positive clones were reckoned only when hybridization could be detected on both sheets. The nucleotide sequence of the present invention was sequenced using a ALF-laser fluorescence sequencer (Pharmacia). The sequence contained all the found amino acid sequence, confirming that the isolated gene indeed encoded the β-1,4-endoglucanase.

Sequence information

SEQ. ID. No. 12 presents the promoter sequence, the enzyme coding sequence, the terminator sequence and the signal sequence and the amino acid sequence of the enzyme of the present invention.

Testing enzyme activity

The purified protein was assayed for endo β-1,4 glucanase activity using Azurine-crosslinked barley β-glucan tablet (trade name: Glucazyme tablets supplied by Megazyme, Australia) by the instructions given by the manufacturer.

The purified enzyme gave a high activity on this substrate. Typically the enzyme has a specific activity of 2250 micromol glucose per min per mg of protein.

Induction of the EglA gene: identification of inducing carbon source

The Table below shows the identification of a number of high and low molecular weight inducers of the glucanase promoter. This analysis was carried out using the full length glucanase promoter of the present invention fused to the *E coli* β-glucuronidase gene

The inducing strength of different carbon sources was determined quantitatively by measuring the intracellular GUS specific activity to hydrolyse p-nitrophenol glucuronide.

CARBON SOURCE (1%)	GUS ACTIVITY (units/mg)- 24 hours
xylose	12.91
xylitol	10.62
arabinose	8.50
arabitol	14.40
glucose	20.25
cellubiose	19.44
xylo-oligomer 70	11.80
glucopyranoside	19.70
methyl-xylopyranoside	12.60
xyloglucan	13.90
pectin	9.70
arabinogalactan	30.20
arabitol + glucose	29.50

Surprisingly glucose, which is normally a potent catabolite repressor, induces the glucanase promoter.

Accordingly, the present invention also relates to a promoter that is inducible by glucose.

In addition, the present invention relates to the use of glucose to induce a promoter.

These aspects of the present invention are different to the teachings of WO 94/04673 which discloses a fungal promoter that is active in the presence of glucose. In this regard, the promoter of the present invention is not only active in the presence of glucose but that it is also inducible by glucose.

One of the advantages of having a glucanase promoter that is inducible by glucose is that the promoter can be used to express a GOI, and thereby be used to prepare a POI (such as an heterologous POI), in a glucose containing environment. This is important because glucose is one of preferred carbon sources for biomass accumulation. In addition, glucose containing media are expected to produce lower amounts of proteases, thereby providing better yields of the POI. In addition, the use of a derepressed promoter in a derepressed host strain will increase the speed and efficiency of reaction media, such as a fermentation reaction medium. In addition, the use of mixed carbon sources during fermentation will allow the efficient induction of this promoter, for example at low levels of glucose and a cheap carbon source (e.g. sugar beet pulp).

Effects of promoter deletions on the regulation of the expression of the glucanase gene

A series of deletion studies, which are shown in Figure 12, were performed. In these studies, the different promoter deletion constructs shown in Figure 12 were fused to the GUS gene. The activity of the reporter gene was assayed qualitatively. The results showed that none of the deletions abolished the inducibility of the glucanase promoter. These results indicate the presence of multiple sites for transcriptional activation and initiation of transcription.

HETEROLOGOUS PROTEIN PRODUCTION USING TRANSFORMANTS OF *ASPERGILLUS NIGER* COMPRISING THE GLUCANASE PROMOTER (GP) AND THE GLUCANASE SIGNAL SEQUENCE (Gss)

Transformation of *Aspergillus Niger*

The protocol for transformation of *A. niger* was based on the teachings of Buxton,F.P., Gwynne D.I., Davis,R.W. 1985 (Transformation of *Aspergillus niger* using the *argB* gene of *Aspergillus nidulans*. Gene 37:207-214). Daboussi,M.J., Djeballi,A., Gerlinger, C., Blaiseau, P.L., Cassan, M., Lebrun, M.H., Parisot, D., Brygoo,Y. 1989 (Transformation of seven species of filamentous fungi using the nitrate reductase gene of

Aspergillus nidulans. Curr. Genet. 15:453-456) and Punt, P.J., van den Hondel, C.A.M.J.J. 1992 (Transformation of filamentous fungi based on hygromycin B and Phleomycin resistance markers. Meth. Enzym. 216:447-457).

For the purification of protoplasts, spores from one PDA (Potato Dextrose Agar - from Difco Lab. Detroit) plate of fresh sporulated N400 (CBS 120.49, Centraalbureau voor Schimmelcultures, Baarn) (7 days old) are washed off in 5-10 ml water. A shake flask with 200 ml Potato Dextrose Broth (difco 0549-17-9, Difco Lab. Detroit) is inoculated with this spore suspension and shaken (250 rpm) for 16-20 hours at 30°C.

The mycelium is harvested using Miracloth paper and 3-4 g wet mycelium are transferred to a sterile petri dish with 10 ml STC (1.2 M sorbitol, 10 mM Tris HCl pH 7.5, 50 mM $CaCl_2$) with 75 mg lysing enzymes (Sigma L-2265) and 4500 units lyticase (Sigma L-8012).

The mycelium is incubated with the enzyme until the mycelium is degraded and the protoplasts are released. The degraded mycelium is then filtered through a sterile 60 μm mesh filter. The protoplasts are harvested by centrifugation 10 min at 2000 rpm in a swing out rotor. The supernatant is discarded and the pellet is dissolved in 8 ml 1.5 M $MgSO_4$ and then centrifuged at 3000 rpm for 10 min.

The upper band, containing the protoplasts is transferred to another tube, using a transfer pipette and 2 ml 0.6 M KCl is added. Carefully 5 ml 30% sucrose is added on the top and the tube is centrifuged 15 min at 3000 rpm.

The protoplasts, lying in the interface band, are transferred to a new tube and diluted with 1 vol. STC. The solution is centrifuged 10 min at 3000 rpm. The pellet is washed twice with STC, and finally solubilized in 1 ml STC. The protoplasts are counted and eventually concentrated before transformation.

For the transformation, 100 µl protoplast solution (10^6-10^7 protoplasts) are mixed with 10 µl DNA solution containing 5- 10 µg DNA and incubated 25 min at room temperature. Then 60 % PEG-4000 is carefully added in portions of 200 µl, 200 µl and 800 µl. The mixture is incubated 20 min at room temperature. 3 ml STC is added to the mixture and carefully mixed. The mixture is centrifugated 3000 rpm for 10 min.

The supernatant is removed and the protoplasts are solubilized in the remaining of the supernatant. 3-5 ml topagarose is added and the protoplasts are quickly spread on selective plates.

Glucanase promoter and heterologous gene expression

Figure 13 shows the expression vector pGPAmy that was used in these studies. This expression vector comprises the glucanase promoter fused to the *Thermomyces lanuginosus* precursor form of the α-amylase gene. Transcription from the promoter is terminated using the xylanase A terminator. This construct was used in a co-transformation experiment with the hygromycin resistance gene as the selectable marker.

The production of α-amylase using four independent transformants containing the expression vector pGPAmy when grown on sugar beet pulp and wheat bran is shown in Figure 14. The α-amylase activity was first detected in the culture medium after 48 hours of growth. A peak of enzyme activity was observed after days 3 and 4.

Glucanase signal sequence & heterologous protein production

For these studies, the expression vector pGPGssAmyHyg was used.

The vector pGPGssAmyHyg is shown in Figure 15. This vector comprises the mature α-amylase gene translationally fused to the glucanase signal peptide (labelled ss). In addition, this vector comprises the promoter of the present invention (labelled EG1 A) and the xylanase A terminator. Transcription from this vector is therefore under the control of the glucanase promoter and termination by the xylanase A terminator.

This construct was used to test *inter alia* the efficiency of the signal peptide in heterologous protein secretion.

Figure 16 shows the results of the induction of α-amylase by use of the construct in strain 6M179 when grown in sugar beet pulp/wheat bran. The results show that the enzyme activity was localised in the culture medium and was first detected after 48 hours of growth. Accumulation of enzyme activity was observed at day 4.

Western Blot

Figure 17 shows a Western blot of proteins from the supernatant of three independent transformants separated by SDS-PAGE and blotted to a membrane. A synthetic peptide with 15 amino acid residues of *T lanuginosus* α-amylase recognised a single band on Western blots of culture supernatants from the transformants.

Antibody Production

Antibodies were raised against the enzyme of the present invention by injecting rabbits with the purified enzyme and isolating the immunoglobulins from antiserum according to procedures described according to N Harboe and A Ingild ("Immunization, Isolation of Immunoglobulins, Estimation of Antibody Titre" In A Manual of Quantitative Immunoelectrophoresis, Methods and Applications, N H Axelsen, *et al* (eds.), Universitetsforlaget, Oslo, 1973) and by T G Cooper ("The Tools of Biochemistry", John Wiley & Sons, New York, 1977).

SUMMARY

Even though it is known that *Aspergillus niger* produces several enzymes which can degrade β-glucan, the present invention provides a novel and inventive β-1,4-endoglucanase, as well as the coding sequence therefor, the termination sequence therefor, the signal sequence therefor, and the promoter for those sequences. An important advantage of the present invention is that the enzyme can be produced in high

amounts. In addition, the promoter and the regulatory sequences (such as the signal sequence and the terminator) can be used to express or can be used in the expression of GOIs in organisms, such as in *A. niger*.

The enzyme of the present invention is advantageous for feed supplements. In addition, it can be used in the brewing industry as it has a high fibre-conversion potential. In addition, there are fewer processing problems when the enzyme is used, particularly with non-starchy polysaccharides. In addition, the enzyme efficiently degrades β-glucans, therefore it can be used advantageously in the brewing industry to lower viscosity and also improve the filterability of beer. This is important as large molecular weight glucans in beer and the like can cause filtration difficulties and give rise to sediments, gels and hazes.

The signal sequence of the present invention is useful for secretion of a POI, such as a heterologous POI, thereby improving the quality and quantity of the POI.

Other modifications of the present invention will be apparent to those skilled in the art without departing from the scope of the invention.

SEQUENCE INFORMATION

ENZYME SEQUENCE
SEQ ID NO: 1:

```
Gln Thr Met Cys Ser Gln Tyr Asp Ser Ala Ser Ser Pro Pro Tyr Ser
 1               5                  10                  15
Val Asn Gln Asn Leu Trp Gly Glu Tyr Gln Gly Thr Gly Ser Gln Cys
            20                  25                  30
Val Tyr Val Asp Lys Leu Ser Ser Ser Gly Ala Ser Trp His Thr Lys
        35                  40                  45
Trp Thr Trp Ser Gly Gly Glu Gly Thr Val Lys Ser Tyr Ser Asn Ser
    50                  55                  60
Gly Leu Thr Phe Asp Lys Lys Leu Val Ser Asp Val Ser Ser Ile Pro
65                  70                  75                  80
Thr Ser Val Thr Trp Ser Gln Asp Asp Thr Asn Val Gln Ala Asp Val
                85                  90                  95
Ser Tyr Asp Leu Phe Thr Ala Ala Asn Ala Asp His Ala Thr Ser Ser
            100                 105                 110
Gly Asp Tyr Glu Leu Met Ile Trp Leu Ala Arg Tyr Gly Ser Val Gln
        115                 120                 125
Pro Ile Gly Lys Gln Ile Ala Thr Ala Thr Val Gly Gly Lys Ser Trp
    130                 135                 140
Glu Val Trp Tyr Gly Thr Ser Thr Gln Ala Gly Ala Glu Gln Lys Thr
145                 150                 155                 160
Tyr Ser Phe Val Ala Gly Ser Pro Ile Asn Ser Trp Ser Gly Asp Ile
                165                 170                 175
Lys Asp Phe Phe Asn Tyr Leu Thr Gln Asn Gln Gly Phe Pro Ala Ser
            180                 185                 190
Ser Gln His Leu Ile Thr Leu Gln Phe Gly Thr Glu Pro Phe Thr Gly
        195                 200                 205
Gly Pro Ala Thr Phe Thr Val Asp Asn Trp Thr Ala Ser Val Asn  *
    210                 215                 220
```

ENZYME CODING SEQUENCE
SEQ ID NO: 2:

CAG ACG ATG TGC TCT CAG TAT GAC AGT GCC TCG AGC CCC GCA TAC TCG
GTG AAC CAG AAC CTC TGG GGC GAA TAC CAG GGC ACT GGC AGC CAG TGT
GTC TAC GTC GAC AAG CTT AGC AGC AGT GGT GCC TCA TGG CAT ACC AAA
TGG ACC TGG AGT GGT GGC GAG GGA ACA GTG AAA AGC TAC TCT AAC TCC
GGC CTT ACG TTT GAC AAG AAG CTA GTC AGC GAT GTG TCA AGC ATT CCC
ACC TCG GTG ACA TGG AGC CAG GAC GAC ACC AAT GTC CAA GCC GAT GTC
TCA TAT GAT CTG TTC ACC GCG GCG AAT GCG GAT CAT GCC ACT TCC AGC
GGT GAC TAT GAG CTT ATG ATT TGG CTT GCC CGC TAC GGC TCA GTC CAG
CCT ATT GGC AAG CAG ATT GCC ACG GCC ACT GTG GGA GGC AAG TCC TGG
GAG GTG TGG TAT GGT ACC AGC ACC CAG GCC GGT GCG GAG CAA AAG ACA
TAT AGC TTC GTG GCA GGA TCT CCT ATC AAC TCG TGG AGT GGG GAC ATT
AAG GAC TTC TTC AAC TAT CTC ACC CAG AAC CAA GGC TTC CCG GCT AGC
TCT CAG CAT TTG ATC ACT CTG CAA TTT GGA ACT GAG CCG TTC ACC GGT
GGC CCG GCA ACC TTC ACG GTT GAC AAC TGG ACC GCT AGT GTC AAC

PROMOTER SEQUENCE

SEQ ID NO: 3:

AATTGAAGCA TTTTGATAGG TTTAAGCCTA ATCAGGATAT TGGATGAGTC GAGTTGCAGA 60
AGTTGAGGAC GGTGGGTGAA ATCGGGGGTT TGATAGGTAG GCAATGCAGG GCGGAACGGG 120
AAGGGTCTAG ACAATTTCTT TCTTTTGGAC AGCTGGTGCG TTTCACTGAG ATTAATAGTA 180
AGCAAACTAC TCGCTCGAAG TCGTAGATGT GCATAATGGA TAACTACAGC CAACCGAAAT 240
CTCCGGGCAG AAGGCCTGGA GGCAGGAGGA AACGTGGATA AGAGAGTAAT GTTTGAGTAT 300
AGATATGTAG GCAAGAAAGG ACTGGGAGGA AGGAAGTATC GCAAACAAGA CAAGTCACTG 360
AATAGGAAAG AATGGGGCCA TCAGAGAAAT GAATCTAAAC GGTAACTGCA GATATTACAT 420
GGAAGAAAAT ACTATGATCC CTAATTGATA TGGTTCCATG GCCCCTGGAG ACTTAAACCT 480
CGTGGTATGA TAAACATATG AGTTACATTC TCGGTAAATC CAACATTACT CCCAAGCTCT 540
GTTGATATTC TCCGATAATT CACCGATAAC CAACCAACCT ACTCCCGTCT AGATCCAATT 600
GGTCTATATG CATAATGGAT ATCGTCAGCA CAGGCAGAAC CCTTTAATTT ATTTCTGGAG 660
ATCCCGTTCT CCACAATGCT TGGTTGCCGA CTGCCACAGA CCATCGCTAA CTTGAAGCGG 720
AAAGTGCTCC GATGAAGGGT CTCATTTTGA AACGGAGGAT TTACATGTCA ATGTTGCAGG 780
CTGGCGTTGA TGATGGCGCA ACCTGCTATA GCTAGTTGGC TTACTTCGTC CTGGCTGCCG 840
TATTGGACAC GGAAAGTCGG ACAATAATAG TGTTAACAGT AAGCGCCATT GATCAGAGTT 900
GATGTATTTA AAGCTGCGTC GTCTGCTGCC CCCTCCGTGT TCGTGTCTTA TTCCAAACAT 960
TCAACCTCTA TTCCTTTCGA AGTCCTTTAG ATCTGCCGTT CCTCTGCTTT ATTGCCCAAC 1020

INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Aspergillus niger

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ser Trp Glu Val Trp Tyr Gly Thr Ser Thr Gln Ala Gly Ala Glu Gln
1 5 10 15

Lys

INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Thr Tyr Ser Phe Val Ala Gly Ser Pro Ile
1 5 10

INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Lys Leu Val Ser Asp Val Ser Ser Ile Pro Thr Ser Val Thr Xaa Ser
1 5 10 15

Gln Asp Asp Thr Asn Xaa Xaa Ala Ala Val Ser Tyr Xaa Leu Phe Thr
20 25 30

Ala Ala Asn
35

INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(v) FRAGMENT TYPE: internal
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Trp Thr Trp Ser Gly Gly Glu Gly Thr Val Lys
1 5 10

INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(v) FRAGMENT TYPE: internal
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Leu Ser Ser Ser Gly Ala Ser Trp His Thr Lys
1 5 10

INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GTN CCR TAC CAN ACY TCC CA 17

INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TGG ACN TGG WSN GGN GG 17

INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 345 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PCR fragment"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GTGGAGTGGT GGCGAGGGAA CAGTGAAAAG CTACTCTAAC TCGGGCCTTA CGTTTGACAA 60

GAAGCTAGTC AGCGATGTGT CAAGCATTCC CACCTCGGTG ACATGGAGCC AGGACGACAC 120

CAATGTCCAA GCCGATGTCT CATATGATCT GTTCACCGCG GCGAATGCGG ATCATGCCAC 180

TTCCAGCGGT GACTATGAGC TTATGATTTG GTATGTGACG TCGTGAACAA GATAGATGGA 240

GGAGGCTAAC GTAACCAGGC TTGCCCGCTA CGGCTCAGTC CAGCCTATTG GCAAGCAGAT 300

TGCCACGGCC ACTGTGGGAG GCAAGTCCTG GGAGGTCTGG TACGG 345

INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2360 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Aspergillus niger

(B) STRAIN: 3M43

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:join(1021..1427, 1476..1708, 1778..1857)

(D) OTHER INFORMATION:/product= "Endoglucanase"

/gene= "eglA"

(ix) FEATURE:

(A) NAME/KEY: exon

(B) LOCATION:1021..1427

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION:1428..1475

(ix) FEATURE:

(A) NAME/KEY: exon

(B) LOCATION:1476..1708

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION:1709..1777

(ix) FEATURE:

(A) NAME/KEY: exon

(B) LOCATION:1778..1954

(ix) FEATURE:

(A) NAME/KEY: sig_peptide

(B) LOCATION:1021..1068

(ix) FEATURE:

(A) NAME/KEY: mat_peptide

(B) LOCATION:join(1069..1427, 1476..1708, 1777..1854)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

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AAGGGTCTAG ACAATTTCTT TCTTTTGGAC AGCTGGTGCG TTTCACTGAG ATTAATAGTA     180
AGCAAACTAC TCGCTCGAAG TCGTAGATGT GCATAATGGA TAACTACAGC CAACCGAAAT     240
CTCCGGGCAG AAGGCCTGGA GGCAGGAGGA AACGTGGATA AGAGAGTAAT GTTTGAGTAT     300
AGATATGTAG GCAAGAAAGG ACTGGGAGGA AGGAAGTATC GCAAACAAGA CAAGTCACTG     360
AATAGGAAAG AATGGGGCCA TCAGAGAAAT GAATCTAAAC GGTAACTGCA GATATTACAT     420
GGAAGAAAAT ACTATGATCC CTAATTGATA TGGTTCCATG GCCCCTGGAG ACTTAAACCT     480
CGTGGTATGA TAAACATATG AGTTACATTC TCGGTAAATC CAACATTACT CCCAAGCTCT     540
GTTGATATTC TCCGATAATT CACCGATAAC CAACCAACCT ACTCCCGTCT AGATCCAATT     600
GGTCTATATG CATAATGGAT ATCGTCAGCA CAGGCAGAAC CCTTTAATTT ATTTCTGGAG     660
ATCCCGTTCT CCACAATGCT TGGTTGCCGA CTGCCACAGA CCATCGCTAA CTTGAAGCGG     720
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CTGGCGTTGA TGATGGCGCA ACCTGCTATA GCTAGTTGGC TTACTTCGTC CTGGCTGCCG     840
TATTGGACAC GGAAAGTCGG ACAATAATAG TGTTAACAGT AAGCGCCATT GATCAGAGTT     900
GATGTATTTA AAGCTGCGTC GTCTGCTGCC CCCTCCGTGT TCGTGTCTTA TTCCAAACAT     960
TCAACCTCTA TTCCTTTCGA AGTCCTTTAG ATCTGCCGTT CCTCTGCTTT ATTGCCCAAC    1020
ATG AAG CTC TCC ATG ACA CTT TCC CTG TTT GCG GCC ACT GCC ATG GGC       1068
Met Lys Leu Ser Met Thr Leu Ser Leu Phe Ala Ala Thr Ala Met Gly
-16 -15                 -10                 -5
CAG ACG ATG TGC TCT CAG TAT GAC AGT GCC TCG AGC CCC CCA TAC TCG       1116
Gln Thr Met Cys Ser Gln Tyr Asp Ser Ala Ser Ser Pro Pro Tyr Ser
  1               5                  10                  15
GTG AAC CAG AAC CTC TGG GGC GAA TAC CAG GGC ACT GGC AGC CAG TGT       1164
Val Asn Gln Asn Leu Trp Gly Glu Tyr Gln Gly Thr Gly Ser Gln Cys
            20                  25                  30
GTC TAC GTC GAC AAG CTT AGC AGC AGT GGT GCC TCA TGG CAT ACC AAA       1212
Val Tyr Val Asp Lys Leu Ser Ser Ser Gly Ala Ser Trp His Thr Lys
        35                  40                  45
TGG ACC TGG AGT GGT GGC GAG GGA ACA GTG AAA AGC TAC TCT AAC TCC       1260
Trp Thr Trp Ser Gly Gly Glu Gly Thr Val Lys Ser Tyr Ser Asn Ser
    50                  55                  60
GGC CTT ACG TTT GAC AAG AAG CTA GTC AGC GAT GTG TCA AGC ATT CCC       1308
Gly Leu Thr Phe Asp Lys Lys Leu Val Ser Asp Val Ser Ser Ile Pro
 65                  70                  75                  80
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ACC TCG GTG ACA TGG AGC CAG GAC GAC ACC AAT GTC CAA GCC GAT GTC      1356
Thr Ser Val Thr Trp Ser Gln Asp Asp Thr Asn Val Gln Ala Asp Val
                 85                  90                  95
TCA TAT GAT CTG TTC ACC GCG GCG AAT GCG GAT CAT GCC ACT TCC AGC      1404
Ser Tyr Asp Leu Phe Thr Ala Ala Asn Ala Asp His Ala Thr Ser Ser
            100                 105                 110
GGT GAC TAT GAG CTT ATG ATT TG  GTATGTGACG TCGTGAACAA                1447
Gly Asp Tyr Glu Leu Met Ile Trp
        115                 120
GATAGATGGA GGAGGCTAAC GTAACCAG G CTT GCC CGC TAC GGC TCA GTC CAG     1500
                                 Leu Ala Arg Tyr Gly Ser Val Gln
                                             125
CCT ATT GGC AAG CAG ATT GCC ACG GCC ACT GTG GGA GGC AAG TCC TGG      1548
Pro Ile Gly Lys Gln Ile Ala Thr Ala Thr Val Gly Gly Lys Ser Trp
    130                 135                 140
GAG GTG TGG TAT GGT ACC AGC ACC CAG GCC GGT GCG GAG CAA AAG ACA      1596
Glu Val Trp Tyr Gly Thr Ser Thr Gln Ala Gly Ala Glu Gln Lys Thr
145                 150                 155                 160
TAT AGC TTC GTG GCA GGA TCT CCT ATC AAC TCG TGG AGT GGG GAC ATT      1644
Tyr Ser Phe Val Ala Gly Ser Pro Ile Asn Ser Trp Ser Gly Asp Ile
                165                 170                 175
AAG GAC TTC TTC AAC TAT CTC ACC CAG AAC CAA GGC TTC CCG GCT AGC      1692
Lys Asp Phe Phe Asn Tyr Leu Thr Gln Asn Gln Gly Phe Pro Ala Ser
            180                 185                 190
TCT CAG CAT TTG ATC A GTGAGTTTTC CTAATTCTAC TAGCGAGCGC               1738
Ser Gln His Leu Ile
        195
CGGCAGTTGA AATTGGTCAC TAACAGAAGT GATGATTAG  CT CTG CAA TTT GGA       1791
                                           Thr Leu Gln Phe Gly
                                               200
ACT GAG CCG TTC ACC GGT GGC CCG GCA ACC TTC ACG GTT GAC AAC TGG      1839
Thr Glu Pro Phe Thr Gly Gly Pro Ala Thr Phe Thr Val Asp Asn Trp
        205                 210                 215
ACC GCT AGT GTC AAC TAA AAGGCTTTAG GCGCGGCTGG GGTAAATAAC            1887
Thr Ala Ser Val Asn  *
    220
AGCTTGTTTC TTCGTTCTAG AACGTCGGGC GTGTAAGAGC TAGAAATCCA CCCACTCTGA    1947
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TTGGAAACAC TCATTCAAGA TCGGTACTCC TCTTCAGCCG AGAAAGGCAC AGATAGTGTA    2007
TCGAATCCAA TCAAATCTAT TTGGTGTTGC TTAAATTCCG AGCCAGTCCT TTCCTTGAAA    2067
GGTAATCCAC CCGTAGCGAT TGATCATTAA CAGATCCGAG TGGTGCTAGG TTAAATTGCT    2127
AACCCGATCC CGCTCCAATT AGCTAGCGCA TCCGGGCAGAT TCAAACTTGA CAGTGGGCCG    2187
GGCATTACCT GAACCTGTAG AAGGAACAGA CCCTTGTCTA GAAATCTCTA AATAGTATAA    2247
GCCGAAACTT GCCCCGGACG TACCCTAAGC TAAGATTGCT CTTCGCATTC CCAGGGGGGT    2307
GAACTCTCTA AAGAGGGAGC ATCGCTTGCC GATGTCTGGT TCGGGGATCA TGA           2360
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(2) INFORMATION FOR SEQ ID NO: 13:

TERMINATOR SEQUENCE

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AAGGCTTTAG GCGCGGCTGG GGTAAATAAC AGCTTGTTTC TTCGTTCTAG   50
AACGTCGGGC GTGTAAGAGC TAGAAATCCA CCCACTCTGA TTGGAAACAC  100
TCATTCAAGA TCGGTACTCC TCTTCAGCCG AGAAAGGCAC AGATAGTGTA  150
TCGAATCCAA TCAAATCTAT TTGGTGTTGC TTAAATTCCG AGCCAGTCCT  200
TTCCTTGAAA GGTAATCCAC CCGTAGCGAT TGATCATTAA CAGATCCGAG  250
TGGTGCTAGG TTAAATTGCT AACCCGATCC CGCTCCAATT AGCTAGCGCA  300
TCCGGGCAGAT TCAAACTTGA CAGTGGGCCG GGCATTACCT GAACCTGTAG  350
AAGGAACAGA CCCTTGTCTA GAAATCTCTA AATAGTATAA GCCGAAACTT  400
GCCCCGGACG TACCCTAAGC TAAGATTGCT CTTCGCATTC CCAGGGGGGT  450
GAACTCTCTA AAGAGGGAGC ATCGCTTGCC GATGTCTGGT TCGGGGATCA  500
TGA                                                     5037
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(2) INFORMATION FOR SEQ ID NO: 14:

SIGNAL SEQUENCE

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ATG AAG CTC TCC ATG ACA CTT TCC CTG TTT GCG GCC ACT GCC ATG GGC      48
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(2) INFORMATION FOR SEQ ID NO: 15:

SIGNAL SEQUENCE

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Met Lys Leu Ser Met Thr Leu Ser Leu Phe Ala Ala Thr Ala Met Gly      16
```

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 26, line 28 and 29

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

The National Collections of Industrial and Marine Bacteria Limited (NCIMB)

Address of depositary institution *(including postal code and country)*

23 St. Machar Drive
Aberdeen
Scotland
AB2 1RY
United Kingdom

Date of deposit	Accession Number
16 JANUARY 1995	NCIMB 40704

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only

☒ This sheet was received with the international application

Authorized officer

J. van Aubel

For International Bureau use only

☐ This sheet was received by the International Bureau on:

Authorized officer

CLAIMS

1. An enzyme obtainable from *Aspergillus*, wherein the enzyme has the following characteristics:

a. a MW of 24,235 D ± 50 D
b. a pI value of about 4
c. glucanase activity

wherein the glucanase activity is endo β-1,4-glucanase activity.

2. An enzyme having sequence shown as SEQ. I.D. No. 1 or a variant, homologue or fragment thereof.

3. An enzyme coded by the nucleotide sequence shown as SEQ. I.D. No. 2 or a variant, homologue or fragment thereof or a sequence complementary thereto.

4. A nucleotide sequence coding for the enzyme according to claim 1.

5. A nucleotide sequence coding for the enzyme according to claim 2.

6. A nucleotide sequence having the sequence shown as SEQ. I.D. No. 2 or a variant, homologue or fragment thereof or a sequence complementary thereto.

7. A nucleotide sequence according to any one of claims 4 to 6 operatively linked to a promoter.

8. A nucleotide sequence according to claim 7 wherein the promoter is the promoter having the sequence shown as SEQ. I.D. No. 3 or a variant, homologue or fragment thereof or a sequence complementary thereto.

9. A promoter having the sequence shown as SEQ. I.D. No. 3 or a variant, homologue or fragment thereof or a sequence complementary thereto.

10. A promoter according to claim 9 operatively linked to a GOI.

11. A promoter according to claim 10 wherein the promoter is operatively linked to a GOI, wherein the GOI comprises a nucleotide sequence according to any one of claims 4-6.

12. A terminator having the nucleotide sequence shown as SEQ. I.D. No. 13 or a variant, homologue or fragment thereof or a sequence complementary thereto.

13. A signal sequence having the nucleotide sequence shown as SEQ. I.D. No. 14 or a variant, homologue or fragment thereof or a sequence complementary thereto.

14. A construct comprising or expressing the invention according to any one of claims 1 to 13.

15. A vector comprising or expressing the invention of any one of claims 1 to 14.

16. A plasmid comprising or expressing the invention of any one of claims 1 to 15.

17. A transgenic organism comprising or expressing the invention according to any one of claims 1 to 16.

18. A transgenic organism according to claim 17 wherein the organism is a fungus.

19. A transgenic organism according to claim 17 wherein the organism is a filamentous fungus, preferably *Aspergillus*.

20. A transgenic organism according to claim 17 wherein the organism is a plant.

21. A transgenic organism according to claim 17 wherein the organism is a yeast.

22. A process of preparing an enzyme according to any one of claims 1 to 3 comprising expressing a nucleotide sequence according to any one of claims 4-8.

23. A process according to claim 22 wherein the enzyme has the sequence shown as SEQ. I.D. No. 1 or a variant, homologue or fragment thereof, and the nucleotide sequence has the sequence shown as SEQ. I.D. No. 2 or a variant, homologue or fragment thereof or a sequence complementary thereto.

24. A process according to claim 22 or claim 23 wherein the expression is controlled (partially or completely) by use of a promoter according to claim 9.

25. A process for expressing a GOI by use of a promoter, wherein the promoter is the promoter according to claim 9.

26. Use of an enzyme according to any one of claims 1 to 3 or prepared by a process according to any one of claims 22 to 25 to degrade a glucan.

27. Plasmid NCIMB 40704, or a nucleotide sequence obtainable therefrom for expressing a glucanase enzyme or for controlling the expression thereof or for controlling the expression of another GOI.

28. A glucanase enzyme having the ability to degrade β-1,4-glucosidic bonds, which is immunologically reactive with an antibody raised against a purified glucanase enzyme having the sequence shown as SEQ. I.D. No. 1.

29. A signal sequence having the sequence shown as SEQ. I.D. No. 15 or a variant, homologue or fragment thereof.

FIGURE 1

AMY 637 PROMOTER
SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA
ORIGINAL SOURCE: *Solanum Tuberosum*
SEQUENCE LENGTH: 2094
SEQUENCE:

```
        10         20         30         40
ATTAAGGGGA GCATAAGTGC AGCTCAGAAA TTCACACCTG
        50         60         70         80
ATATTTTCCC AAAGCCCTCA AAAATGTGAA CAAATCTGCT
        90        100        110        120
AAAATGTCAG TCAGAAGGAC TGTTCTTTTA GGTTTTCTTC
       130        140        150        160
TCTCGAGTCA CGAAATCAGA TAATATGATA AGAAATTATG
       170        180        190        200
GAGGATTTAT AATGTATCTG TCTGTTCTTA GGTATAATTA
       210        220        230        240
TGTGTTCCTT TATGATGTAG TAATGGAATT CTGGGCTTAT
       250        260        270        280
ATTAAAGGAA CTGAATATAA ATGTTCGCAT TTTAACTGCG
       290        300        310        320
GAGACTTCGA GTTAGAGCCT TATAATTATG TCTTATCATT
       330        340        350        360
TTATACTGAG ATCATATTAC AGATGATGAA AGCTGACATT
       370        380        390        400
GCATTAGTTA TTCTGTTTTA TACAAGTCAT GTAACTGCTG
       410        420        430        440
CTTGTGAGTT GTGACTGTAA GATAAATTGA TTCAGCCTTC
       450        460        470        480
TGTGGCATTA GCGGAGATCT GATTATACTC TCATCGTCTT
       490        500        510        520
ATCTAAGTTG CTCATGCAAC TTTGTCCTTG ATAGTTGGCT
       530        540        550        560
AATACTACAA CTGGAATTAA GTGTAGTTAT TCGAAATCTC
       570        580        590        600
TGTTGGAAGT TGCTAAGTGC TTAAGTGCTG GTTATTGTAA
       610        620        630        640
ACCCCATCCG AGTTATTATA CAGCATCTGG CTGATGAAAT
       650        660        670        680
GCTGCTCATT TGCAATGGTG ACATAACCAA ATGTTAGTAA
       690        700        710        720
AACATACTAG CTGGTTGAAT GTTAGATGAT TGTTCAACGT
       730        740        750        760
TACATCTCAC AGAAACCTTA TTATGGATTG ACATGTTAGT
       770        780        790        800
TGATCCGAAA GATCCTTCTT TTAAATGCCA AAGCTTGTTA
       810        820        830        840
CAGATTTGAG GAGTTCTTTT ACTTTCTTTT GTTATATCTA
       850        860        870        880
TTTCCCATTC ATTTTGACGT TCAGCCTCAC AGATGTTGTC
       890        900        910        920
ATACTTAGAA ATGTGCGTAT ATATATAGAG AGAGAGAGAT
       930        940        950        960
AGAGTGAAAT GATTATATAG TCGAAGATTA CGAAACTTGA
```

```
       970         980         990        1000
CATTGAGACA TCTGTGATTG TTTGAAATTT ATGTATATAT
      1010        1020        1030        1040
CTGTAGCATT AGAAACTATA AGAGTTGTTA GCTTCACTTG
      1050        1060        1070        1080
TCTTATTGTT GTGCTCAAAG CAACTTCATC ATACAGTATG
      1090        1100        1110        1120
GTTTTTATAT GCTCTTCCAT TATCACCGAA CCTTATGATT
      1130        1140        1150        1160
ATGTGTACGA GCTTATAATA TTACTGATGG TGATTCAGTA
      1170        1180        1190        1200
TTATGATTAT GTCCTCCATT AATTATTCTG TTTCATACAA
      1210        1220        1230        1240
GTCGTGTAAT TTGCTGTTTG TGATTGTACG ATAAATTGAT
      1250        1260        1270        1280
TCAACCTTCT GCGGTGTTGG TTGAAGTTCA AGTAAATTAG
      1290        1300        1310        1320
CTTTATTTAT CATAGTAGCA TTTGATTATT GATGCTCTGT
      1330        1340        1350        1360
AGCTAATGAT AAGCCATTGA AGGGAAGCAG AAATGGTAAA
      1370        1380        1390        1400
GCTTTCTAAA ATGAATCTAC GAATGGATGA TAAAGTTAAT
      1410        1420        1430        1440
GAATATTGTT GATACTTCTG CAATCAGATT ATGAGTTACT
      1450        1460        1470        1480
GAGTCTACTG TTTTTTAAGC CTGTTTCAGA TGATCGATCA
      1490        1500        1510        1520
TCAACAACAA CATATTCAGT GTAGTAGACA TGATCGATCA
      1530        1540        1550        1560
CTTTCTAATT TTCGATTATG CACCCTCTTT TCTCCAATTT
      1570        1580        1590        1600
GGTCGTCTTC TTTTTTTCAT GATGTCACTG AATTATTCTC
      1610        1620        1630        1640
TGGTCGTCCC CACCATTCAG GAAGTCACTT CGAGCATAAT
      1650        1660        1670        1680
GTGAAAACAT CCACATTTTT CAAATCCAGC AGAATTTTCA
      1690        1700        1710        1720
TCAAACGGGA TTCAACATTT ACTACATGTA TACACTCTGA
      1730        1740        1750        1760
AGTCTGAATC CACTAATTCT AGATGGTGCA TCTGTGCCCC
      1770        1780        1790        1800
CACACTTGTG AAAGCTTATT CTCAATTTTT TATTTTCCAA
      1810        1820        1830        1840
CAACTTGAAT TCAGACCACA CAACTCCCGT GTCTTGTACG
      1850        1860        1870        1880
GTCAGCATCT GAGTGGAGAA CTCAATTAAG TGACTTTAAC
      1890        1900        1910        1920
GTCGAGTTCT ATAGTAAACA ACCCCTATAT CTTTTTTCAA
      1930        1940        1950        1960
GCATGTTAAG ATTGCGAACA CACTGAAATT TCCAGGTCGT
      1970        1980        1990        2000
TAATCTTGTA CCCAGTGTGT GTACTTTTAA AAAAAAAAGT
      2010        2020        2030        2040
CAGTTTTTTA GTCTCTAAAA CACATTTAAA TAGAGTTTAT
      2050        2060        2070        2080
TTGCCATCTT TTGTTCCTCA TACTAGACTT CGGAGTCAAC
      2090
ACAACACAAC AACA
```

FIGURE 1 CONTINUED

FIGURE 2

AMY 351 PROMOTER
SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA (genomic)
ORIGINAL SOURCE: *Solanum tuberosum*
SEQUENCE LENGTH: 1734 bp
STRANDEDNESS: Double
TOPOLOGY: Linear
SEQUENCE:

```
        10         20         30         40
TCTTTAAGTT GTTTGCTTGA TTTTTCTTCT TCAATCTTCT
        50         60         70         80
ATATTTAATT CGTTTTAGCT TCAAACTTCT TCAATTTTAT
        90        100        110        120
TTCAATTTAA TTCTACAAAA AAAATCTCTA TTTAGCACCA
       130        140        150        160
TTCATAAAAT TCATGCTCAA AATGGGCAAA CATAAATAAT
       170        180        190        200
AAATGTGAAG TAAATAATGG ATTAAAATAT ATATTTTTGG
       210        220        230        240
GCCTCACATC AACCTTCATA ATTCTTGAAT GAATGAATGA
       250        260        270        280
TAGACTTCAT AATTTTTTAA CCTATACATA TAAGAAAATT
       290        300        310        320
GAGAGTAACT CAAATAACAA GTTGTAGTAT CACATCTTTA
       330        340        350        360
CTATTTGATA ACATTATGAA GGTGATTATA CATTACGTAA
       370        380        390        400
CATTTCTTTT AAAAATATGT AAGCAAATTT ACTTTTTAAC
       410        420        430        440
TTATCATTGA TCTTCATGGT TTTGTCATAA ATCTCAAAGT
       450        460        470        480
TATCATATTT TATATAGCTA TTTGAAAGTA ATTTTATTTT
       490        500        510        520
TACTCATCAT TGAGTGATGC TTTTATTATA ATACTAGTAA
       530        540        550        560
GTTTTATTTA TTATTTTCTT TTAGGGGTGA ATTGTATAAT
       570        580        590        600
ATAATAAAAA ATATATTTTT AGAAATAATG ATTCTTTTAT
       610        620        630        640
TATTAAAAAG TTAAGATATT AGATTATTTA TGCTTGTATA
       650        660        670        680
ATAATGAACG AAGTTTTATT TTCTATGAGT TTCATTAATC
       690        700        710        720
ATGTTTGTAA TTATTTCAAA TTTTGATGTA TTTTTATAAT
       730        740        750        760
TTTGTATTAT TATATTATTA TACTATATTT AAAAATTTAA
       770        780        790        800
AGATCCATAG GGCTTACGCC CCACGTCAAG AGGCTTGCGC
       810        820        830        840
CTTTCCCTAA ATTAAGTAAA ACTCTTCGCC TCATGCCTTA
       850        860        870        880
CGCCTCCGCC TTTTAAAACA CTGATTCGTT TCCTCATATA
       890        900        910        920
GCTTGAGGCG AAAATATTTA ATAAAAACAC TTCTTAATTT
       930        940        950        960
GTTTATATGT TCAATTGAAC ATGTCCGTGA TTAGAAAATT
```

FIGURE 2 CONTINUED

```
       970         980         990        1000
AAATTAAATT CAATGACAAA TTTAATAATT TGACACAAAA
      1010        1020        1030        1040
TTTATGAAAA AAATATCAAA ATATAAAGAA ATATTTTTTT
      1050        1060        1070        1080
TGAAATGGAT TAAAAAGAAA AAAAAAACAA ATAAATTGAA
      1090        1100        1110        1120
CCGGGATAAG TTGGTTGTTT AATTGATTAT TGATTATGAT
      1130        1140        1150        1160
CTCAATTTGA CATTTTGCGC GATCTTTCGA CCTCAATTCG
      1170        1180        1190        1200
TATGAACTGA CACTACGCCA ATGGACAGTC GCCGTCGTCA
      1210        1220        1230        1240
CCGCCACCGC ACTATTCTCG ACGCGTCGTC TATCTCCTCC
      1250        1260        1270        1280
ACCCCACAGC CGTCAATTCC AAGCTTCCAA TGAACCGTTG
      1290        1300        1310        1320
CCATGTGTCA CTGCCTATTC ACCGCGAAAC ATGAATATCA
      1330        1340        1350        1360
CTGACGAACG ATTTCGGAGC GGAACGAATC CAGAAAATGG
      1370        1380        1390        1400
ATTACTTTCT ATAAATTCCT CGAATCTCAA CTCCATTTCG
      1410        1420        1430        1440
TAAAAATAAA ATTAAAAATA TTGTTTCTTT TTGTATTTCT
      1450        1460        1470        1480
TTTTGTATTT CTGGTTTATG TGGTGATCGA ATTTTCAATT
      1490        1500        1510        1520
TTTTTACTGG TAGTGATTCC TACTTTTCTT CAATTGCATT
      1530        1540        1550        1560
TCTCCTTTTT CCATTTCACG GTTGAGAATT CATGATTCCT
      1570        1580        1590        1600
TATCAGAGGA ATCGATCCGA TTTGACTAAT TTCACTTTTC
      1610        1620        1630        1640
GTCTGTATAA ATACCAGAGT ATCTAGGTTG AGGAACGTAA
      1650        1660        1670        1680
TTTCAAGCTG CGATCGGCTT TTTCCCCTGA ACGAGCAAAC
      1690        1700        1710        1720
ACAGGTTGTG GGTTCGAGTT AGCAAGGGAC GTATAATCTC
      1730
AACTACAATC CATT
```

FIGURE 3 5/31
α-AMYLASE CODING SEQUENCE
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2017 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(A) LENGTH: 475 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

```
ATG AAG TCT CTC GCC GCA ATT GCT GCT CTG CTG TCG CCC ACA CTG GTC      48
Met Lys Ser Leu Ala Ala Ile Ala Ala Leu Leu Ser Pro Thr Leu Val
-18         -15                 -10                 -5

CGG GCA GCG ACT CCG GAT GAG TGG AAA GCT CAG TCG ATC TAT TTC ATG      96
Arg Ala Ala Thr Pro Asp Glu Trp Lys Ala Gln Ser Ile Tyr Phe Met
        1               5                  10

CTG ACG GAC CGG TTT GCG CGT ACC GAC AAT TCG ACC ACG GCT CCC TGT     144
Leu Thr Asp Arg Phe Ala Arg Thr Asp Asn Ser Thr Thr Ala Pro Cys
 15                  20                  25                  30

GAC ACC ACT GCC GGG GTATGCAACT AACCCTGTGT TTCTCTTCCC GGGACGTACA     199
Asp Thr Thr Ala Gly
                35

AGGGGTCTTC TCCATGCTAA CCGTGCACAT GCAG AAA TAT TGC GGG GGA ACA       251
                                      Lys Tyr Cys Gly Gly Thr
                                                  40

TGG CGA GGT ATC ATC AAC AAC GTAAGTGGCT TCTGATTTTC GCTCAATAAT       302
Trp Arg Gly Ile Ile Asn Asn
            45

CTTCGTCGCG TGACTTTATT TCCTAG CTG GAT TAC ATC CAG GAT ATG GGC TTC    355
                             Leu Asp Tyr Ile Gln Asp Met Gly Phe
                                 50                  55

ACA GCT ATC TGG ATA ACT CCA GTG ACA GCC CAG TGG GAC GAC GAT GTG     403
uThr Ala Ile Trp Ile Thr Pro Val Thr Ala Gln Trp Asp Asp Asp Val
        60                  65                  70

GAT GCG GCA GAT GCA ACG TCG TAT CAC GGT TAT TGG CAG AAA GAC  CT     450
Asp Ala Ala Asp Ala Thr Ser Tyr His Gly Tyr Trp Gln Lys Asp  Leu
    75                  80                  85

GTGCGCAACC CTGCTCCATG GATCGCTGGC TGCAAACTCG TGCTGATCGG TGATTTTTTT   510

TTTTTTTTTT TTGAAACAG A TAC TCT CTG AAT TCG AAA TTC GGC ACT GCC      560
                      Tyr Ser Leu Asn Ser Lys Phe Gly Thr Ala
                      90                  95
```

FIGURE 3 CONTINUED

```
GAT GAC TTG AAA GCC CTG GCT GAT GCC CTT CAC GCC CGT GGG ATG CTT          508
Asp Asp Leu Lys Ala Leu Ala Asp Ala Leu His Ala Arg Gly Met Leu
100                 105                 110                 115

CTC ATG GTC GAC GTC GTG GCT AAT CAC TTT GTACGGACCA TCTACATACC           558
Leu Met Val Asp Val Val Ala Asn His Phe
                120                 125

TGGGAAACGC GAAGAAGGAA AAAAAAAAAA AGGCGCACGC TAACATTTCG CGTTTAG          715

GGC TAC GGC GGT TCT CAT AGC GAG GTG GAT TAC TCG ATC TTC AAT CCT          763
Gly Tyr Gly Gly Ser His Ser Glu Val Asp Tyr Ser Ile Phe Asn Pro
                130                 135                 140

CTG AAC AGC CAG GAT TAC TTC CAC CCG TTC TGT CTC ATT GAG GAC TAC          811
Leu Asn Ser Gln Asp Tyr Phe His Pro Phe Cys Leu Ile Glu Asp Tyr
        145                 150                 155

GAC AAC CAG GAA GAA GTC GAA CAA TGC TGG CTG GCC GAT ACT CCG ACG          859
Asp Asn Gln Glu Glu Val Glu Gln Cys Trp Leu Ala Asp Thr Pro Thr
    160                 165                 170

ACA TTG CCC GAC GTG GAC ACC ACC AAT CCT CAG GTT CGG ACG TTT TTC          907
Thr Leu Pro Asp Val Asp Thr Thr Asn Pro Gln Val Arg Thr Phe Phe
175                 180                 185

AAC GAC TGG ATC AAG AGC CTG GTG GCG AAC TAC TCC  A GTATGATTGT           954
Asn Asp Trp Ile Lys Ser Leu Val Ala Asn Tyr Ser
190                 195                 200

TCCCGCGGTA ACGCTTTAGG GCTTGCTCTA ACTGAAATCG ACAG  TC GAT GGT CTG        1009
                                                 Ile Asp Gly Leu
                                                             205

CGC GTC GAC ACC GTT AAG CAC GTG GAG AAA GAT TTC TGG CCC GAC TTC         1057
Arg Val Asp Thr Val Lys His Val Glu Lys Asp Phe Trp Pro Asp Phe
            210                 215                 220

AAC GAA GCT GCT GCG TGT ACC GTC GGC GAG GTG TTC AAC GGT GAC CCA         1105
Asn Glu Ala Ala Ala Cys Thr Val Gly Glu Val Phe Asn Gly Asp Pro
        225                 230                 235

GCG TAC ACC TGC CCA TAC CAG GAA GTG CTG GAT GGC GTT CTG AAC TAT         1153
Ala Tyr Thr Cys Pro Tyr Gln Glu Val Leu Asp Gly Val Leu Asn Tyr
    240                 245                 250

CCG  AT  GTGAGTGATT CCGAAAGTTC CATCGATCAG GCTTTCTGAC GCATGAGAAC         1208
Pro  Ile
     255
```

FIGURE 3 CONTINUED

```
AGC TAC TAT CCT GCG CTT GAT GCA TTC AAG TCT GTC GGC GGC AAT CTC                1256
    Tyr Tyr Pro Ala Leu Asp Ala Phe Lys Ser Val Gly Gly Asn Leu
                260                 265                 270

GGC GGC TTG GCT CAG GCC ATC ACC ACC GTG CAG GAG AGC TGC AAG GAT               1304
Gly Gly Leu Ala Gln Ala Ile Thr Thr Val Gln Glu Ser Cys Lys Asp
            275                 280                 285

TCC AAT CTG CTC GGC AAT TTC CTT GAG AAT CAC GAC ATT GCT CGC TTT               1352
Ser Asn Leu Leu Gly Asn Phe Leu Glu Asn His Asp Ile Ala Arg Phe
        290                 295                 300

GCT  TC  GTATGGACAC TCTTTTTTGAA GCCCTCATCG ATTGGGGATG CTGACACGGA              1407
Ala  Ser

CAACAACAAC AG G TAC ACG GAT GAC CTT GCT CTC GCC AAG AAT GGT CTC                1456
              Tyr Thr Asp Asp Leu Ala Leu Ala Lys Asn Gly Leu
              305                 310                 315

GCT TTC ATC ATC CTC TCG GAT GGT ATT CCG ATC ATC TAC ACG GGC CAG               1504
Ala Phe Ile Ile Leu Ser Asp Gly Ile Pro Ile Ile Tyr Thr Gly Gln
            320                 325                 330

GAG CAG CAC TAC GCC GGT GAT CAC GAT CCC ACA AAT CGT GAG GCC GTC               1552
Glu Gln His Tyr Ala Gly Asp His Asp Pro Thr Asn Arg Glu Ala Val
        335                 340                 345

TGG CTG TCT GGC TAC AAT ACC GAC GCC GAG CTG TAC CAG TTC ATC AAG               1600
Trp Leu Ser Gly Tyr Asn Thr Asp Ala Glu Leu Tyr Gln Phe Ile Lys
    350                 355                 360

AAG GCC AAT GGC ATC CGC AAC TTG GCT ATC AGC CAG AAC CCG GAA TTC               1648
Lys Ala Asn Gly Ile Arg Asn Leu Ala Ile Ser Gln Asn Pro Glu Phe
365                 370                 375                 380

ACC TCC TCC AAG GTGAGTACAA TAACAAACTT TTCGAAAAAT TTTTCACCGG                   1700
Thr Ser Ser Lys

AGAAAACCTA AGATTCGGCT AACAAAACAA AAAAAAAAAA G ACC AAG GTC ATC                 1753
                                              Thr Lys Val Ile
                                              385

TAC CAA GAC GAT TCG ACC CTT GCC ATT AAC CGG GGC GGC GTC GTT ACT               1801
Tyr Gln Asp Asp Ser Thr Leu Ala Ile Asn Arg Gly Gly Val Val Thr
    390                 395                 400

GTC CTG AGC AAT GAA GGC GCC TCC GGG GAG ACC GGG ACT GTC TCC ATT               1849
Val Leu Ser Asn Glu Gly Ala Ser Gly Glu Thr Gly Thr Val Ser Ile
405                 410                 415                 420
```

FIGURE 3 CONTINUED


```
CCG GGA ACT GGC TTC GAG GCC GGC ACG GAA TTG ACT GAT GTC ATC TCC          1897
Pro Gly Thr Gly Phe Glu Ala Gly Thr Glu Leu Thr Asp Val Ile Ser
                425                 430                 435

TGC AAG ACC GTG ACT GCG GGG GAC AGC GGG GCG GTC GAC GTG CCC TTG          1945
Cys Lys Thr Val Thr Ala Gly Asp Ser Gly Ala Val Asp Val Pro Leu
            440                 445                 450

TCG GGC GGA CTG CCA AGC GTG CTC TAT CCC AGC TCC CAG CTG GCC AAG          1993
Ser Gly Gly Leu Pro Ser Val Leu Tyr Pro Ser Ser Gln Leu Ala Lys
        455                 460                 465

AGT GGT CTG TGT GCG TCG GCG TGA                                          2017
Ser Gly Leu Cys Ala Ser Ala
    470                 475
```

FIGURE 4

α-AMYLASE CODING SEQUENCE
SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA
ORIGINAL SOURCE: *Solanum Tuberosum*
SEQUENCE LENGTH: 1570
SEQUENCE:

```
        10         20         30         40
TGTGGTGATC GAATTTTCAA TTTTTTTACT GAGTATCTAG
        50         60         70         80
GTTGAGGAAC GTAATTTCAA GCTGCGATCG GCTTTTTCCC
        90        100        110        120
CTGAACGAGC AAACACAGGT TGTGGGTTCG AGTTAGCAAG
       130        140        150        160
GGACGTATAA TCTCAACTAC AATCCATTAT GGCGCTTGAT
       170        180        190        200
GAAAGTCAGC AGTCTGATCC ATTGGTTGTG ATACGCAATG
       210        220        230        240
GAAAGGAGAT CATATTGCAG GCATTCGACT GGGAATCTCA
       250        260        270        280
TAAACATGAT TGGTGGCTAA ATTTAGATAC GAAAGTTCCT
       290        300        310        320
GATATTGCAA AGTCTGGTTT CACAACTGCT TGGCTGCCTC
       330        340        350        360
CGGTGTGTCA GTCATTGGCT CCTGAAGGTT ACCTTCCACA
       370        380        390        400
GAACCTTTAT TCTCTCAATT CTAAATATGG TTCTGAGGAT
       410        420        430        440
CTCTTAAAAG CTTTACTTAA TAAGATGAAG CAGTACAAAG
       450        460        470        480
TTAGAGCGAT GGCGGACATA GTCATTAACC ACCGTGTTGG
       490        500        510        520
GACTACTCAA GGGCATGGTG GAATGTACAA CCGCTATGAT
       530        540        550        560
GGAATTCCTA TGTCTTGGGA TGAACATGCT ATTACATCTT
       570        580        590        600
GCACTGGTGG AAGGGGTAAC AAAAGCACTG GAGACAACTT
       610        620        630        640
TAATGGAGTT CCAAATATAG ATCATACACA ATCCTTTGTT
       650        660        670        680
CGGAAAGATC TCATTGACTG GATGCGGTGG CTAAGATCCT
       690        700        710        720
CTGTTGGCTT CCAAGATTTT CGTTTTGATT TTGCCAAAGG
       730        740        750        760
TTATGCTTCA AAGTATGTAA AGGAATATAT CGAGGGAGCT
       770        780        790        800
GAGCCAATAT TTGCAGTTGG AGAATACTGG GACACTTGCA
       810        820        830        840
ATTACAAGGG CAGCAATTTG GATTACAACC AAGATAGTCA
       850        860        870        880
CAGGCAAAGA ATCATCAATT GGATTGATGG CGCGGGACAA
       890        900        910        920
CTTTCAACTG CATTCGATTT TACAACAAAA GCAGTCCTTC
```

FIGURE 4 CONTINUED

```
       930        940        950        960
AGGAAGCAGT CAAAGGAGAA TTCTGGCGTT TGCGTGACTC
       970        980        990       1000
TAAGGGGAAG CCCCCAGGAG TTTTAGGATT GTGGCCTTCA
      1010       1020       1030       1040
AGGGCTGTCA CTTTTATTGA TAATCACGAC ACTGGATCAA
      1050       1060       1070       1080
CTCAGGCGCA TTGGCCTTTC CCTTCACGTC ATGTTATGGA
      1090       1100       1110       1120
GGGCTATGCA TACATTCTTA CACACCCAGG GATACCATCA
      1130       1140       1150       1160
GTTTTCTTTG ACCATTTCTA CGAATGGGAT AATTCCATGC
      1170       1180       1190       1200
ATGACCAAAT TGTAAAGCTG ATTGCTATTC GGAGGAATCA
      1210       1220       1230       1240
AGGCATACAC AGCCGTTCAT CTATAAGAAT TCTTGAGGCA
      1250       1260       1270       1280
CAGCCAAACT TATACGCTGC AACCATTGAT GAAAAGGTTA
      1290       1300       1310       1320
GCGTGAAGAT TGGGGACGGA TCATGGAGCC CTGCTGGGAA
      1330       1340       1350       1360
AGAGTGGACT CTCGCGACCA GTGGCCATCG CTATGCAGTC
      1370       1380       1390       1400
TGGCAGAAGT AATCTTACAG CTATTCCGTT ACTTAATATA
      1410       1420       1430       1440
TTAGTAGAAA TATATATGTT TTAAACCCGA GCACCTACTT
      1450       1460       1470       1480
CTAACACTAG ATCCGCCTCT ACAGGCTTGG ATGGAGTGAT
      1490       1500       1510       1520
GAGTTTTTTT TTCCTGTTCA TTAGACATTG CAACATGGGA
      1530       1540       1550       1560
TGTATGTTTT GTTAATAAAA GTGTTCTTGA TCAATGCAAT
      1570
GTAATAAGGG
```

FIGURE 5

SEQUENCE: Nucleotide sequence of a cDNA encoding the large subunit of ADP-glucose pyrophosphorylase from barley seed endosperm (bepl10)
SEQUENCE TYPE: NUCLEIC ACID
MOLECULE TYPE: DNA
ORIGINAL SOURCE: BARLEY
SEQUENCE LENGTH: 2037
STRANDEDNESS: DOUBLE
TOPOLOGY: LINEAR

```
1     ACGACCACCT CCGAACTCAA CGCCTCCACG GACCATCTCT
41    CTCCTCTCCC CTCCCCTCAC CACCACCACC ACCACCACCC
81    CTTCTCCCTC CCTGCATTTG ATTCGTTCAT ATTCATCCGT
121   CGCTTGCCCG GTCGCCACCC CGTCGATCCC TCACCCCGCC
161   GTCCCCGGCA GTTGCAGGTG GACTGCTAAT GTCATCGATG
201   CAGTTCAGCA GCGTGCTGCC CCTGGAGGGC AAGGCGTGCG
241   TTTCCCCAGT CAGGAGAGAG GGATCGGCCT GCGAGCGCCT
281   CAAGATCGGG GACAGCAGCA GCATCAGGCA CGAGAGAGCG
321   TCCAGGAGGA TGTGCAACGG CGGCGCAGGG GCCCCGCCGC
361   CACCGGTGCG CAGTGCGTGC TCACCTCCGA CGCCAGCCCG
401   GCCGACACCC TTGTTCTCCG GACGTCCTTC CGGAGGAATT
      ACGCCGATCC GAACGAGGTC GCGGCCGTCG GTCGCGGCCG
      TCATACTCGG CGGCGGCACC GGGACTCAGC TCTTCCCGCT
      CACAAGCACA AGGGCCACAC CTGCTGTTCC TATTGGAGGA
      TGTTACAGGC TCATCGATAT TCCCATGAGC AACTGCTTCA
601   ACAGTGGCAT CAACAAGATA TTCGTCATGA CCCAGTTCAA
      CTCGGCATCT CTCAATCGCC ACATTCACCG CACCTACCTC
      GGCGGGGGAA TCAATTTCAC TGATGGATCT GTTGAGGTAT
      TGGCCGCGAC ACAAATGCCT GGGGAGGCTG CTGGATGGTT
      CCGCGGAACA GCGGATGCCG TCAGAAAATT TATCTGGGTG
801   CTTGAGGACT ACTATAAGCA TAAATCCATA GAGCACATTT
      TGATCTTGTC GGGCGATCAG CTTTATCGCA TGGATTACAT
      GGAGCTTGTG CAGAAACATG TGGATGACAA TGCTGACATT
      ACTTTATCAT GTGCCCCTGT TGGAGAGAGC CGGGCATCTG
      AGTACGGGCT AGTGAAGTTC GACAGTTCAG GCCGTGTGAT
1001  CCAGTTTTCT GAGAAGCCAA AGGGCGACGA TCTGGAAGCG
      ATGAAAGTGG ATACCAGTTT TCTCAATTTC GCCATAGACG
      ACCCTGCTAA ATATCCATAC ATTGCTTCGA TGGGAGTTTA
      TGTCTTCAAG AGAGATGTTC TGCTGAACCT TCTAAAGTCA
      AGATACGCAG AACTACATGA CTTTGGGTCT GAAATCCTCC
1201  CGAGAGCTCT GCATGATCAC AATGTACAGG CATATGTCTT
      CACTGACTAC TGGGAGGACA TTGGAACAAT CAGATCCTTC
      TTCGATGCGA ACATGGCCCT CTGCGAACAG CCTCCAAAGT
      TTGAATTTTA TGATCCAAAA ACCCCCTTCT TCACTTCGCC
      TCGGTACTTA CCGCCAACAA AGTCAGACAA GTGCAGGATC
1401  AAAGAAGCGA TCATTTCGCA CGGCTGCTTC TTGCGTGAAT
      GCAAAATCGA GCACTCCATC ATCGGCGTTC GTTCACGCCT
      AAACTCCGGA AGCGAGCTCA AGAACGCGAT GATGATGGGC
      GCGGACTCGT ACGAGACCGA GGACGAGATC TCGAGGCTGA
      TGTCTGAGGG CAAGGTTCCC ATCGGCGTCG GGGAGAACAC
1601  AAAGATCAGC AACTGCATCA TCGACATGAA CGCGAGGATA
```

FIGURE 5 CONTINUED

```
       GGAAGGGACG TGGTCATCTC AAACAAGGAG GGGGTGCAAG
       AAGCCGACAG GCCGGAGGAA GGGTACTACA TCAGGTCCGG
       GATCGTGGTG ATCCAGAAGA ACGCGACCAT CAAGGACGGC
       ACCGTCGTGT AGGGCGTGCC GGGTCGGCGC GACGGGGTTC
1801   TGCGACAACC TGTGCGCTGC GTCGGTCGTC ATCATCTTCT
       CAAACTCCGG GACTGAAGAA GTGATCCGGG GACGGGAGAC
       GTTTCAAGCT TGAATGACTG AGACTGAAAG TGAAGGCGCA
       GCAGAGGCAG GCAGCATTAG TAGTAAGTAG TAAGTAAGTA
       GCAGTGGAAC AAAGTAATAG TCGTTCGTTT TTCCCCTGTA
2001   ATAAATAAGA GGCTGTGTGT TGAGGTAAAA AAAAAAA
```

FIGURE 6

SEQUENCE: Nucleotide sequence of a cDNA encoding the small subunit of ADP-glucose pyrophosphorylase from barley seed endosperm (beps)
SEQUENCE TYPE: NUCLEIC ACID
MOLECULE TYPE: DNA
ORIGINAL SOURCE: BARLEY
SEQUENCE LENGTH: 1822
STRANDEDNESS: DOUBLE
TOPOLOGY: LINEAR
COMMENT: The "." at 1569 denotes a purine.

```
1     AAAAGTGAAC TCACACATCA CTCAATATCT ATATCCTTCC
      ATTTTATATC CCTCGGTGAT GGATGTACCT TTGGCATCTA
      AAGTTCCCTT GCCCTCCCCT TCCAAGCATG AACAATGCAA
      CGTTTATAGT CATAAGAGCT CATCGAAGCA TGCAGATCTC
      AATCCCCATG CTATTGATAG TGTTCTCGGT ATCATTCTTG
201   GAGGTGGTGC AGGGACTAGA TTGTATCCCC TGACGAAGAA
      GCGTGCAAAG CCTGCAGTGC CATTGGGTGC CAACTACAGG
      CTTATTGATA TTCCTGTCAG TAATTGTCTG AACAGCAACA
      TATCAAAGAT CTATGTGCTT ACACAGTTCA ACTCAGCTTC
      TCTTAATCGT CATCTCTCAC GAGCCTATGG GAGCAACATT
401   GGAGGTTACA AGAATGAAGG ATTTGTTGAA GTCCTTGCTG
      CACAGCAGAG CCCAGATAAC CCTGACTGGT TCCAGGGTAC
      TGCAGATGCT GTAAGGCAGT ACTTGTGGCT ATTCGAGGAG
      CATAATGTTA TGGAGTATCT AATTCTTGCT GGAGATCACC
      TGTACCGAAT GGACTATGAA AAGTTTATTC AGGCACACAG
601   AGAAACGGAT GCTGATATTA CTGTTGCTGC CTTGCCCATG
      GATGAGGAAC GTGCAACTGC ATTTGGCCTT ATGAAAATCG
      ATGAAGAAGG GAGGATAATT GAATTCGCAG AGAAACCAAA
      AGGAGAACAG TTGAAAGCTA TGATGGTTGA TACGACCATA
      CTTGGCCTTG AAGATGCGAG GGCAAAGGAA ATGCCTTATA
801   TTGCTAGCAT GGGTATCTAT GTTATTAGCA AACATGTGAT
      GCTTCAGCTT CTCCGTGAGC AATTTCCTGG AGCTAATGAC
      TTCGGAAGTG AAGTTATCCC TGGTGCAACT AGCACTGGCA
      TGAGGGTACA AGCATACCTA TACGACGGTT ACTGGGAAGA
      TATTGGTACA ATTGAGGCAT TCTATAATGC AAATTTGGGA
1001  ATTACCAAAA AACCAATACC TGATTTCAGT TTCTATGACC
      GTTCTGCTCC CATTTACACA CAACCTCGAC ACTTGCCTCC
      TTCAAAGGTT CTTGATGCTG ATGTGACAGA CAGTGTAATT
      GGTGAAGGAT GTGTTATTAA AAACTGCAAG ATACACCATT
      CAGTAGTTGG ACTCCGTTCC TGCATATCTG AAGGTGCAAT
1201  AATAGAGGAC ACGTTGCTAA TGGGTGCGGA CTACTATGAG
      ACTGAAGCTG ATAAGAAACT CCTTGCTGAA AAAGGTGGCA
      TTCCCATTGG TATTGGAAAG AATTCACACA TCAAAAGAGC
      AATCATTGAC AAGAATGCTC GTATTGGAGA TAACGTGATG
      ATAATCAATG TTGACAATGT TCAAGAAGCG GCGAGGGAGA
1401  CAGATGGATA CTTCATCAAA AGTGGCATCG TAACTGTGAT
      CAAGGATGCT TTACTCCCTA GTGGAACAGT CATATGAAGC
      AGATGTGAAA TGTATGCCAA AAGACAGGGC TACTTGCGTC
      AGTCTGGAAT CAACCAACAA GGCCGCGAAG GAGATCATAA
      AATAAAAA.G GAGTGCCATG CGAGTCACTT CTACACCCTT
1601  TTCCCCCCTT GATGTATTAG GAACTGTGAT GTACAAGCAA
```

FIGURE 6 CONTINUED

```
      CTGTGATGCA CTTACGCGAA GTGCCCCTGG ATTCAGCTTT
      CTCTTTGCTT GTAACTGGTT TCCAGCAGAC CATGCTATTT
      GTTGTATGGT TCGTGCAAAA CCTTGCGATG CTTTATATAT
      GCTTTATATA TAAACAAGAT GAATCCCCGC GCGTTGCTGC
2001  GGCACAAAAA AAAAAAAAAA AA
```

FIGURE 7

α-GLUCAN LYASE CODING SEQUENCE
SEQUENCE TYPE: NUCLEIC ACID
MOLECULE TYPE: DNA (GENOMIC)
ORIGINAL SOURCE: FUNGALLY INFECTED ALGAE
SEQUENCE LENGTH: 3267 BP
STRANDEDNESS: DOUBLE
SEQUENCE:

```
                10        20        30        40        50        60
                |         |         |         |         |         |
   1 ATGTTTTCAA CCCTTGCGTT TGTCGCACCT AGTGCGCTGG GAGCCAGTAC CTTCGTAGGG
  61 GCGGAGGTCA GGTCAAATGT TCGTATCCAT TCCGCTTTTC CAGCTGTGCA CACAGCTACT
 121 CGCAAAACCA ATCGCCTCAA TGTATCCATG ACCGCATTGT CCGACAAACA AACGGCTACT
 181 GCGGGTAGTA CAGACAATCC GGACGGTATC GACTACAAGA CCTACGATTA CGTCGGAGTA
 241 TGGGGTTTCA GCCCCCTCTC CAACACGAAC TGGTTTGCTG CCGGCTCTTC TACCCCGGGT
 301 GGCATCACTG ATTGGACGGC TACAATGAAT GTCAACTTCG ACCGTATCGA CAATCCGTCC
 361 ATCACTGTCC AGCATCCCGT TCAGGTTCAG GTCACGTCAT ACAACAACAA CAGCTACAGG
 421 GTTCGCTTCA ACCCTGATGG CCCTATTCGT GATGTGACTC GTGGGCCTAT CCTCAAGCAG
 481 CAACTAGATT GGATTCGAAC GCAGGAGCTG TCAGAGGGAT GTGATCCCGG AATGACTTTC
 541 ACATCAGAAG GTTTCTTGAC TTTTGAGACC AAGGATCTAA GCGTCATCAT CTACGGAAAT
 601 TTCAAGACCA GAGTTACGAG AAAGTCTGAC GGCAAGGTCA TCATGGAAAA TGATGAAGTT
 661 GGAACTGCAT CGTCCGGGAA CAAGTGCCGG GGATTGATGT TCGTTGATAG ATTATACGGT
 721 AACGCTATCG CTTCCGTCAA CAAGAACTTC CGCAACGACG CGGTCAAGCA GGAGGGATTC
 781 TATGGTGCAG GTGAAGTCAA CTGTAAGTAC CAGGACACCT ACATCTTAGA ACGCACTGGA
 841 ATCGCCATGA CAAATTACAA CTACGATAAC TTGAACTATA ACCAGTGGGA CCTTAGACCT
 901 CCGCATCATG ATGGTGCCCT CAACCCAGAC TATTATATTC CAATGTACTA CGCAGCACCT
 961 TGGTTGATCG TTAATGGATG CGCCGGTACT TCGGAGCAGT ACTCGTATGG ATGGTTCATG
1021 GACAATGTCT CTCAATCTTA CATGAATACT GGAGATACTA CCTGGAATTC TGGACAAGAG
1081 GACCTGGCAT ACATGGGCGC GCAGTATGGA CCATTTGACC AACATTTTGT TTACGGTGCT
1141 GGGGGTGGGA TGGAATGTGT GGTCACAGCG TTCTCTCTTC TACAAGGCAA GGAGTTCGAG
1201 AACCAAGTTC TCAACAAACG TTCAGTAATG CCTCCGAAAT ACGTCTTTGG TTTCTTCCAG
1261 GGTGTTTTCG GGACTTCTTC CTTGTTGAGA GCGCATATGC CAGCAGGTGA GAACAACATC
1321 TCAGTCGAAG AAATTGTAGA AGGTTATCAA AACAACAATT TCCCTTTCGA GGGGCTCGCT
1381 GTGGACGTGG ATATGCAAGA CAACTTGCGG GTGTTCACCA CGAAGGGCGA ATTTTGGACC
1441 GCAAACAGGG TGGGTACTGG CGGGGATCCA AACAACCGAT CGGTTTTTGA ATGGGCACAT
1501 GACAAAGGCC TTGTTTGTCA GACAAATATA ACTTGCTTCC TGAGGAATGA TAACGAGGGG
1561 CAAGACTACG AGGTCAATCA GACGTTAAGG GAGAGGCAGT TGTACACGAA GAACGACTCC
1621 CTGACGGGTA CGGATTTTGG AATGACCGAC GACGGCCCCA GCGATGCGTA CATCGGTCAT
1681 CTGGACTATG GGGGTGGAGT AGAATGTGAT GCACTTTTCC CAGACTGGGG ACGGCCTGAC
1741 GTGGCCGAAT GGTGGGGAAA TAACTATAAG AAACTGTTCA GCATTGGTCT CGACTTCGTC
1801 TGGCAAGACA TGACTGTTCC AGCAATGATG CCGCACAAAA TTGGCGATGA CATCAATGTG
1861 AAACCGGATG GGAATTGGCC GAATGCGGAC GATCCGTCCA ATGGACAATA CAACTGGAAG
1921 ACGTACCATC CCCAAGTGCT TGTAACTGAT ATGCGTTATG AGAATCATGG TCGGGAACCG
1981 ATGGTCACTC AACGCAACAT TCATGCGTAT ACACTGTGCG AGTCTACTAG GAAGGAAGGG
2041 ATCGTGGAAA ACGCAGACAC TCTAACGAAG TTCCGCCGTA GCTACATTAT CAGTCGTGGT
2101 GGTTACATTG GTAACCAGCA TTTCGGGGGT ATGTGGGTGG GAGACAACTC TACTACATCA
2161 AACTACATCC AAATGATGAT TGCCAACAAT ATTAACATGA ATATGTCTTG GTTGGCTTTC
2221 GTCGGCTCCG ACATTGGAGG ATTCACCTCA TACGACAATG AGAATCAGCG AACGCCGTCT
2281 ACCGGGGACT TGATGGTGAG GTATGTGCAG GCGGGCTGCC TGTTGCCGTG GTTCAGGAAC
2341 CACTATGATA GGTGGATCGA GTCCAAGGAC CACGGAAAGG ACTACCAGGA GCTGTACATG
2401 TATCCGAATG AAATGGATAC GTTGAGGAAG TTCGTTGAAT TCGCTTATGA GTGGCAGGAA
2461 GTGTTGTACA CGGCCATGTA CCAGAATGCG GCTTTCGGAA AGCCGATTAT CAAGGCTGCT
2521 TCGATGTACA ATAACGACTC AAACGTTCGC AGGGCGCAGA ACGATCATTT CGTTGTTGGT
2581 GGACATGATG GATATCGCAT TGTGTGCGCG CCTGTTGTGT GGGAGAATTT GACCGAACGC
```

FIGURE 7 CONTINUED

```
2641 GAATTGTACT TGCCCGTGCT GACCCAATGG TACAAATTCG GTCCCGACTT TGACACCAAG
2701 CCTCTGGAAG GAGCGATGAA CGGAGGGGAC CGAATTTACA ACTACCCTGT ACCGCAAAGT
2761 GAATCACCAA TCTTCGTGAG AGAAGGTGCG ATTCTCCCTA CCCGCTACAC GTTGAACGGT
2821 GAAAACAAAT CATTGAACAC GTACACGGAC GAAGATCCGT TGGTGTTTGA AGTATTCCCC
2881 CTCGGAAACA ACCGTGCCGA CGGTATGTGT TATCTTGATG ATGGCGGTGT GACCACCAAT
2941 GCTGAAGACA ATGGCAAGTT CTCTGTCGTC AAGGTGGCAG CGGAGCAGGA TGGTGGTACG
3001 GAGACGATAA CGTTTACGAA TGATTGCTAT GAGTACGTTT TCGGTGGACC GTTCTACGTT
3061 CGAGTGCGCG GCGCTCAGTC GCCGTCGAAC ATCCACGTGT CTTCTGGAGC GGGTTCTCAG
3121 GACATGAAGG TGAGCTCTGC CACTTCCAGG GCTGCGCTGT TCAATGACGG GGAGAACGGT
3181 GATTTCTGGG TTGACCAGGA GACAGATTCT CTGTGGCTGA AGTTGCCCAA CGTTGTTCTC
3241 CCGGACGCTG TGATCACAAT TACCTAA
```

FIGURE 8

α-GLUCAN LYASE CODING SEQUENCE
SEQUENCE TYPE: NUCLEIC ACID
MOLECULE TYPE: DNA (GENOMIC)
ORIGINAL SOURCE: FUNGALLY INFECTED ALGAE
SEQUENCE LENGTH: 3276 BP
STRANDEDNESS: DOUBLE
SEQUENCE:

```
                10         20         30         40         50         60
                |          |          |          |          |          |
     1 ATGTATCCAA CCCTCACCTT CGTGGCGCCT AGTGCGCTAG GGGCCAGAAC TTTCACGTGT
    61 GTGGGCATTT TTAGGTCACA CATTCTTATT CATTCGGTTG TTCCAGCGGT GCGTCTAGCT
   121 GTGCGCAAAA GCAACCGCCT CAATGTATCC ATGTCCGCTT TGTTCGACAA ACCGACTGCT
   181 GTTACTGGAG GGAAGGACAA CCCGGACAAT ATCAATTACA CCACTTATGA CTACGTCCCT
   241 GTGTGGCGCT TCGACCCCCT CAGCAATACG AACTGGTTTG CTGCCGGATC TTCCACTCCC
   301 GGCGATATTG ACGACTGGAC GGCGACAATG AATGTGAACT TCGACCGTAT CGACAATCCA
   361 TCCTTCACTC TCGAGAAACC GGTTCAGGTT CAGGTCACGT CATACAAGAA CAATTGTTTC
   421 AGGGTTCGCT TCAACCCTGA TGGTCCTATT CGCGATGTGG ATCGTGGGCC TATCCTCCAG
   481 CAGCAACTAA ATTGGATCCG GAAGCAGGAG CAGTCGAAGG GGTTTGATCC TAAGATGGGC
   541 TTCACAAAAG AAGGTTTCTT GAAATTTGAG ACCAAGGATC TGAACGTTAT CATATATGGC
   601 AATTTTAAGA CTAGAGTTAC GAGGAAGAGG GATGGAAAAG GGATCATGGA GAATAATGAA
   661 GTGCCGGCAG GATCGTTAGG GAACAAGTGC CGGGGATTGA TGTTTGTCGA CAGGTTGTAC
   721 GGCACTGCCA TCGCTTCCGT TAATGAAAAT TACCGCAACG ATCCCGACAG GAAAGAGGGG
   781 TTCTATGGTG CAGGAGAAGT AAACTGCGAG TTTTGGGACT CCGAACAAAA CAGGAACAAG
   841 TACATCTTAG AACGAACTGG AATCGCCATG ACAAATTACA ATTATGACAA CTATAACTAC
   901 AACCAGTCAG ATCTTATTGC TCCAGGATAT CCTTCCGACC CGAACTTCTA CATTCCCATG
   961 TATTTTGCAG CACCTTGGGT AGTTGTTAAG GGATGCAGTG GCAACAGCGA TGAACAGTAC
  1021 TCGTACGGAT GGTTTATGGA TAATGTCTCC CAAACTTACA TGAATACTGG TGGTACTTCC
  1081 TGGAACTGTG GAGAGGAGAA CTTGGCATAC ATGGGAGCAC AGTGCGGTCC ATTTGACCAA
  1141 CATTTTGTGT ATGGTGATGG AGATGGTCTT GAGGATGTTG TCCAAGCGTT CTCTCTTCTG
  1201 CAAGGCAAAG AGTTTGAGAA CCAAGTTCTG AACAAACGTG CCGTAATGCC TCCGAAATAT
  1261 GTGTTTGGTT ACTTTCAGGG AGTCTTTGGG ATTGCTTCCT TGTTGAGAGA GCAAAGACCA
  1321 GAGGGTGGTA ATAACATCTC TGTTCAAGAG ATTGTCGAAG GTTACCAAAG CAATAACTTC
  1381 CCTTTAGAGG GGTTAGCCGT AGATGTGGAT ATGCAACAAG ATTTGCGCGT GTTCACCACG
  1441 AAGATTGAAT TTTGGACGGC AAATAAGGTA GGCACCGGGG GAGACTCGAA TAACAAGTCG
  1501 GTGTTTGAAT GGGCACATGA CAAAGGCCTT GTATGTCAGA CGAATGTTAC TTGCTTCTTG
  1561 AGAAACGACA ACGGCGGGGC AGATTACGAA GTCAATCAGA CATTGAGGGA GAAGGGTTTG
  1621 TACACGAAGA ATGACTCACT GACGAACACT AACTTCGGAA CTACCAACGA CGGGCCGAGC
  1681 GATGCGTACA TTGGACATCT GGACTATGGT GGCGGAGGGA ATTGTGATGC ACTTTTCCCA
  1741 GACTGGGGTC GACCGGGTGT GGCTGAATGG TGGGGTGATA ACTACAGCAA GCTCTTCAAA
  1801 ATTGGTCTGG ATTTCGTCTG GCAAGACATG ACAGTTCCAG CTATGATGCC ACACAAAGTT
  1861 GGCGACGCAG TCGATACGAG ATCACCTTAC GGCTGGCCGA ATGAGAATGA TCCTTCGAAC
  1921 GGACGATACA ATTGGAAATC TTACCATCCA CAAGTTCTCG TAACTGATAT GCGATATGAG
  1981 AATCATGGAA GGGAACCGAT GTTCACTCAA CGCAATATGC ATGCGTACAC ACTCTGTGAA
  2041 TCTACGAGGA AGGAAGGGAT TGTTGCAAAT GCAGACACTC TAACGAAGTT CCGCCGCAGT
  2101 TATATTATCA GTCGTGGAGG TTACATTGGC AACCAGCATT TTGGAGGAAT GTGGGTTGGA
  2161 GACAACTCTT CCTCCCAAAG ATACCTCCAA ATGATGATCG CGAACATCGT CAACATGAAC
  2221 ATGTCTTGCC TTCCACTAGT TGGGTCCGAC ATTGGAGGTT TTACTTCGTA TGATGGACGA
  2281 AACGTGTGTC CCGGGGATCT AATGGTAAGA TTCGTGCAGG CGGGGTTGCT ACTACCGTGG
  2341 TTCAGAAACC ACTATGGTAG GTTGGTCGAG GGCAAGCAAG AGGGAAAATA CTATCAAGAA
  2401 CTGTACATGT ACAAGGACGA GATGGCTACA TTGAGAAAAT TCATTGAATT CCGTTACCGC
  2461 TGGCAGGAGG TGTTGTACAC TGCTATGTAC CAGAATGCGG CTTTCGGGAA ACCGATTATC
  2521 AAGGCAGCTT CCATGTACGA CAACGACAGA AACGTTCGCG GCGCACAGGA TGACCACTTC
  2581 CTTCTCGGCG GACACGATGG ATATCGTATT TTGTGTGCAC CTGTTGTGTG GGAGAATACA
```

FIGURE 8 CONTINUED

```
2641 ACCAGTCGCG ATCTGTACTT GCCTGTGCTG ACCAAATGGT ACAAATTCGG CCCTGACTAT
2701 GACACCAAGC GCCTGGATTC TGCGTTGGAT GGAGGGCAGA TGATTAAGAA CTATTCTGTG
2761 CCACAAAGCG ACTCTCCGAT ATTTGTGAGG GAAGGAGCTA TTCTCCCTAC CCGCTACACG
2821 TTGGACGGTT CGAACAAGTC AATGAACACG TACACAGACA AAGACCCGTT GGTGTTTGAG
2881 GTATTCCCTC TTGGAAACAA CCGTGCCGAC GGTATGTGTT ATCTTGATGA TGGCGGTATT
2941 ACTACAGATG CTGAGGACCA TGGCAAATTC TCTGTTATCA ATGTCGAAGC CTTACGGAAA
3001 GGTGTTACGA CGACGATCAA GTTTGCGTAT GACACTTATC AATACGTATT TGATGGTCCA
3061 TTCTACGTTC GAATCCGTAA TCTTACGACT GCATCAAAAA TTAACGTGTC TTCTGGAGCG
3121 GGTGAAGAGG ACATGACACC GACCTCTGCG AACTCGAGGG CAGCTTTGTT CAGTGATGGA
3181 GGTGTTGGAG AATACTGGGC TGACAATGAT ACGTCTTCTC TGTGGATGAA GTTGCCAAAC
3241 CTGGTTCTGC AAGACGCTGT GATTACCATT ACGTAG
```

FIGURE 9

α-GLUCAN LYASE CODING SEQUENCE
SEQUENCE TYPE: NUCLEIC ACID
MOLECULE TYPE: DNA (GENOMIC)
ORIGINAL SOURCE: FUNGUS
SEQUENCE LENGTH: 3201 BP
STRANDEDNESS: DOUBLE
SEQUENCE:

```
        10         20         30         40         50         60
ATGGCAGGAT TTTCTGATCC TCTCAACTTT TGCAAAGCAG AAGACTACTA CAGTGTTGCG
        70         80         90        100        110        120
CTAGACTGGA AGGGCCCTCA AAAAATCATT GGAGTAGACA CTACTCCTCC AAAGAGCACC
       130        140        150        160        170        180
AAGTTCCCCA AAAACTGGCA TGGAGTGAAC TTGAGATTCG ATGATGGGAC TTTAGGTGTG
       190        200        210        220        230        240
GTTCAGTTCA TTAGGCCGTG CGTTTGGAGG GTTAGATACG ACCCTGGTTT CAAGACCTCT
       250        260        270        280        290        300
GACGAGTATG GTGATGAGAA TACGAGGACA ATTGTGCAAG ATTATATGAG TACTCTGAGT
       310        320        330        340        350        360
AATAAATTGG ATACTTATAG AGGTCTTACG TGGGAAACCA AGTGTGAGGA TTCGGGAGAT
       370        380        390        400        410        420
TTCTTTACCT TCTCATCCAA GGTCACCGCC GTTGAAAAAT CCGAGCGGAC CCGCAACAAG
       430        440        450        460        470        480
GTCGGCGATG GCCTCAGAAT TCACCTATGG AAAAGCCCTT TCCGCATCCA AGTAGTGCGC
       490        500        510        520        530        540
ACCTTGACCC CTTTGAAGGA TCCTTACCCC ATTCCAAATG TAGCCGCAGC CGAAGCCCGT
       550        560        570        580        590        600
GTGTCCGACA AGGTCGTTTG GCAAACGTCT CCCAAGACAT TCAGAAAGAA CCTGCATCCG
       610        620        630        640        650        660
CAACACAAGA TGCTAAAGGA TACAGTTCTT GACATTGTCA AACCTGGACA TGGCGAGTAT
       670        680        690        700        710        720
GTGGGGTGGG GAGAGATGGG AGGTATCCAG TTTATGAAGG AGCCAACATT CATGAACTAT
       730        740        750        760        770        780
TTTAACTTCG ACAATATGCA ATACCAGCAA GTCTATGCCC AAGGTGCTCT CGATTCTCGC
       790        800        810        820        830        840
GAGCCACTGT ACCACTCGGA TCCCTTCTAT CTTGATGTGA ACTCCAACCC GGAGCACAAG
       850        860        870        880        890        900
AATATCACGG CAACCTTTAT CGATAACTAC TCTCAAATTG CCATCGACTT TGGAAAGACC
       910        920        930        940        950        960
AACTCAGGCT ACATCAAGCT GGGAACCAGG TATGGTGGTA TCGATTGTTA CGGTATCAGT
       970        980        990       1000       1010       1020
GCGGATACGG TCCCGGAAAT TGTACGACTT TATACAGGTC TTGTTGGACG TTCAAAGTTG
      1030       1040       1050       1060       1070       1080
AAGCCCAGAT ATATTCTCGG GGCCCATCAA GCCTGTTATG GATACCAACA GGAAAGTGAC
      1090       1100       1110       1120       1130       1140
TTGTATTCTG TGGTCCAGCA GTACCGTGAC TGTAAATTTC CACTTGACGG GATTCACGTC
      1150       1160       1170       1180       1190       1200
GATGTCGATG TTCAGGACGG CTTCAGAACT TTCACCACCA ACCCCACACAC TTTCCCTAAC
      1210       1220       1230       1240       1250       1260
CCCAAAGAGA TGTTTACTAA CTTGAGGAAT AATGGAATCA AGTGCTCCAC CAATATCACT
      1270       1280       1290       1300       1310       1320
CCTGTTATCA GCATTAACAA CAGAGAGGGT GGATACAGTA CCCTCCTTGA GGGAGTTGAC
```

FIGURE 9 CONTINUED

```
      1330        1340        1350        1360        1370        1380
AAAAAATACT TTATCATGGA CGACAGATAT ACCGAGGGAA CAAGTGGGAA TGCGAAGGAT
      1390        1400        1410        1420        1430        1440
GTTCGGTACA TGTACTACGG TGGTGGTAAT AAGGTTGAGG TCGATCCTAA TGATGTTAAT
      1450        1460        1470        1480        1490        1500
GGTCGGCCAG ACTTTAAAGA CAACTATGAC TTCCCCGCGA ACTTCAACAG CAAACAATAC
      1510        1520        1530        1540        1550        1560
CCCTATCATG GTGGTGTGAG CTACGGTTAT GGGAACGGTA GTGCAGGTTT TTACCCGGAC
      1570        1580        1590        1600        1610        1620
CTCAACAGAA AGGAGGTTCG TATCTGGTGG GGAATGCAGT ACAAGTATCT CTTCGATATG
      1630        1640        1650        1660        1670        1680
GGACTGGAAT TTGTGTGGCA AGACATGACT ACCCCAGCAA TCCACACATC ATATGGAGAC
      1690        1700        1710        1720        1730        1740
ATGAAAGGGT TGCCCACCCG TCTACTCGTC ACCTCAGACT CCGTCACCAA TGCCTCTGAG
      1750        1760        1770        1780        1790        1800
AAAAAGCTCG CAATTGAAAC TTGGGCTCTC TACTCCTACA ATCTCCACAA AGCAACTTGG
      1810        1820        1830        1840        1850        1860
CATGGTCTTA GTCGTCTCGA ATCTCGTAAG AACAAACGAA ACTTCATCCT CGGGCGTGGA
      1870        1880        1890        1900        1910        1920
AGTTATGCCG GAGCCTATCG TTTTGCTGGT CTCTGGACTG GGGATAATGC AAGTAACTGG
      1930        1940        1950        1960        1970        1980
GAATTCTGGA AGATATCGGT CTCTCAAGTT CTTTCTCTGG GCCTCAATGG TGTGTGCATC
      1990        2000        2010        2020        2030        2040
GCGGGGTCTG ATACGGGTGG TTTTGAACCC TACCGTGATG CAAATGGGGT CGAGGAGAAA
      2050        2060        2070        2080        2090        2100
TACTGTAGCC CAGAGCTACT CATCAGGTGG TATACTGGTT CATTCCTCTT GCCGTGGCTC
      2110        2120        2130        2140        2150        2160
AGGAACCATT ATGTCAAAAA GGACAGGAAA TGGTTCCAGG AACCATACTC GTACCCCAAG
      2170        2180        2190        2200        2210        2220
CATCTTGAAA CCCATCCAGA ACTCGCAGAC CAAGCATGGC TCTATAAATC CGTTTTGGAG
      2230        2240        2250        2260        2270        2280
ATCTGTAGGT ACTATGTGGA GCTTAGATAC TCCCTCATCC AACTACTTTA CGACTGCATG
      2290        2300        2310        2320        2330        2340
TTTCAAAACG TaGTCGACGG TATGCCAATC ACCAGATCTA TGCTCTTGAC CGATACTGAG
      2350        2360        2370        2380        2390        2400
GATACCACCT TCTTCAACGA GAGCCAAAAG TTCCTCGACA ACCAATATAT GGCTGGTGAC
      2410        2420        2430        2440        2450        2460
GACATTCTTG TTGCACCCAT CCTCCACAGT CGCAAAGAAA TTCCAGGCGA AAACAGAGAT
      2470        2480        2490        2500        2510        2520
GTCTATCTCC CTCTTTACCA CACCTGGTAC CCCTCAAATT TGAGACCATG GGACGATCAA
      2530        2540        2550        2560        2570        2580
GGAGTCGCTT TGGGGAATCC TGTCGAAGGT GGTAGTGTCA TCAATTATAC TGCTAGGATT
      2590        2600        2610        2620        2630        2640
GTTGCACCCG AGGATTATAA TCTCTTCCAC AGCGTGGTAC CAGTCTACGT TAGAGAGGGT
      2650        2660        2670        2680        2690        2700
GCCATCATCC CGCAAATCGA AGTACGCCAA TGGACTGGCC AGGGGGGAGC CAACCGCATC
      2710        2720        2730        2740        2750        2760
AAGTTCAACA TCTACCCTGG AAAGGATAAG GAGTACTGTA CCTATCTTGA TGATGGTGTT
      2770        2780        2790        2800        2810        2820
AGCCGTGATA GTGCGCCGGA AGACCTCCCA CAGTACAAAG AGACCCACGA ACAGTCGAAG
      2830        2840        2850        2860        2870        2880
GTTGAAGGCG CGGAAATCGC AAAGCAGATT GGAAAGAAGA CGGGTTACAA CATCTCAGGA
      2890        2900        2910        2920        2930        2940
```

FIGURE 9 CONTINUED

```
ACCGACCCAG AAGCAAAGGG TTATCACCGC AAAGTTGCTG TCACACAAAC GTCAAAAGAC
      2950       2960       2970       2980       2990       3000
AAGACGCGTA CTGTCACTAT TGAGCCAAAA CACAATGGAT ACGACCCTTC CAAAGAGGTG
      3010       3020       3030       3040       3050       3060
GGTGATTATT ATACCATCAT TCTTTGGTAC GCACCAGGTT TCGATGGCAG CATCGTCGAT
      3070       3080       3090       3100       3110       3120
GTGAGCAAGA CGACTGTGAA TGTTGAGGGT GGGGTGGAGC ACCAAGTTTA TAAGAACTCC
      3130       3140       3150       3160       3170       3180
GATTTACATA CGGTTGTTAT CGACGTGAAG GAGGTGATCG GTACCACAAA GAGCGTCAAG
      3190       3200
ATCACATGTA CTGCCGCTTA A
```

FIGURE 10

α-GLUCAN LYASE CODING SEQUENCE
SEQUENCE TYPE: NUCLEIC ACID
MOLECULE TYPE: DNA (GENOMIC)
ORIGINAL SOURCE: FUNGUS
SEQUENCE LENGTH: 3213 BP
STRANDEDNESS: DOUBLE
SEQUENCE:

```
        10          20          30          40          50          60
ATGGCAGGAT TATCCGACCC TCTCAATTTC TGCAAAGCAG AGGACTACTA CGCTGCTGCC
        70          80          90         100         110         120
AAAGGCTGGA GTGGCCCTCA GAAGATCATT CGCTATGACC AGACCCCTCC TCAGGGTACA
       130         140         150         160         170         180
AAAGATCCGA AAAGCTGGCA TGCGGTAAAC CTTCCTTTCG ATGACGGGAC TATGTGTGTA
       190         200         210         220         230         240
GTGCAATTCG TCAGACCCTG TGTTTGGAGG GTTAGATATG ACCCCAGTGT CAAGACTTCT
       250         260         270         280         290         300
GATGAGTACG GCGATGAGAA TACGAGGACT ATTGTACAAG ACTACATGAC TACTCTGGTT
       310         320         330         340         350         360
GGAAACTTGG ACATTTTCAG AGGTCTTACG TGGGTTTCTA CGTTGGAGGA TTCGGGCGAG
       370         380         390         400         410         420
TACTACACCT TCAAGTCCGA AGTCACTGCC GTGGACGAAA CCGAACGGAC TCGAAACAAG
       430         440         450         460         470         480
GTCGGCGACG GCCTCAAGAT TTACCTATGG AAAAATCCCT TTCGCATCCA GGTAGTGCGT
       490         500         510         520         530         540
CTCTTGACCC CCCTGGTGGA CCCTTTCCCC ATTCCCAACG TAGCCAATGC CACAGCCCGT
       550         560         570         580         590         600
GTGGCCGACA AGGTTGTTTG GCAGACGTCC CCGAAGACGT TCAGGAAAAA CTTGCATCCG
       610         620         630         640         650         660
CAGCATAAGA TGTTGAAGGA TACAGTTCTT GATATTATCA AGCCGGGGCA CGGAGAGTAT
       670         680         690         700         710         720
GTGGGTTGGG GAGAGATGGG AGGCATCGAG TTTATGAAGG AGCCAACATT CATGAATTAT
       730         740         750         760         770         780
TTCAACTTTG ACAATATGCA ATATCAGCAG GTCTATGCAC AAGGCGCTCT TGATAGTCGT
       790         800         810         820         830         840
GAGCCGTTGT ATCACTCTGA TCCCTTCTAT CTCGACGTGA ACTCCAACCC AGAGCACAAG
       850         860         870         880         890         900
AACATTACGG CAACCTTTAT CGATAACTAC TCTCAGATTG CCATCGACTT TGGGAAGACC
       910         920         930         940         950         960
AACTCAGGCT ACATCAAGCT GGGTACCAGG TATGGCGGTA TCGATTGTTA CGGTATCAGC
       970         980         990        1000        1010        1020
GCGGATACGG TCCCGGAGAT TGTGCGACTT TATACTGGAC TTGTTGGGCG TTCGAAGTTG
      1030        1040        1050        1060        1070        1080
AAGCCCAGGT ATATTCTCGG AGCCCACCAA GCTTGTTATG GATACCAGCA GGAAAGTGAC
      1090        1100        1110        1120        1130        1140
TTGCATGCTG TTGTTCAGCA GTACCGTGAC ACCAAGTTTC CGCTTGATGG GTTGCATGTC
      1150        1160        1170        1180        1190        1200
GATGTCGACT TCAAGGACAA TTTCAGAACG TTACCACTA ACCCGATTAC GTTCCCTAAT
      1210        1220        1230        1240        1250        1260
CCCAAAGAAA TGACCAA TCTAAGGAAC AATGGAATCA AGTGTTCCAC CAACATCACC
      1270        1280        1290        1300        1310        1320
```

FIGURE 10 CONTINUED

CCTGTTATCA GTATCAGAGA TCGCCCGAAT GGGTACAGTA CCCTCAATGA GGGATATGAT
1330 1340 1350 1360 1370 1380
AAAAAGTACT TCATCATGGA TGACAGATAT ACCGAGGGGA CAAGTGGGGA CCCGCAAAAT
1390 1400 1410 1420 1430 1440
GTTCGATACT CTTTTTACGG CGGTGGGAAC CCGGTTGAGG TTAACCCTAA TGATGTTTGG
1450 1460 1470 1480 1490 1500
GCTCGGCCAG ACTTTGGAGA CAATTATGAC TTCCCTACGA ACTTCAACTG CAAAGACTAC
1510 1520 1530 1540 1550 1560
CCCTATCATG GTGGTGTGAG TTACGGATAT GGGAATGGCA CTCCAGGTTA CTACCCTGAC
1570 1580 1590 1600 1610 1620
CTTAACAGAG AGGAGGTTCG TATCTGGTGG GGATTGCAGT ACGAGTATCT CTTCAATATG
1630 1640 1650 1660 1670 1680
GGACTAGAGT TTGTATGGCA AGATATGACA ACCCCAGCGA TCCATTCATC ATATGGAGAC
1690 1700 1710 1720 1730 1740
ATGAAAGGGT TGCCCACCCG TCTGCTCGTC ACCGCCGACT CAGTTACCAA TGCCTCTGAG
1750 1760 1770 1780 1790 1800
AAAAAGCTCG CAATTGAAAG TTGGGCTCTT TACTCCTACA ACCTCCATAA AGCAACCTTC
1810 1820 1830 1840 1850 1860
CACGGTCTTG GTCGTCTTGA GTCTCGTAAG AACAAACGTA ACTTCATCCT CGGACGTGGT
1870 1880 1890 1900 1910 1920
AGTTACGCCG GTGCCTATCG TTTTGCTGGT CTCTGGACTG GAGATAACGC AAGTACGTGG
1930 1940 1950 1960 1970 1980
GAATTCTGGA AGATTTCGGT CTCCCAAGTT CTTTCTCTAG GTCTCAATGG TGTGTGTATA
1990 2000 2010 2020 2030 2040
GCGGGGTCTG ATACGGGTGG TTTTGAGCCC GCACGTACTG AGATTGGGGA GGAGAAATAT
2050 2060 2070 2080 2090 2100
TGCAGTCCGG AGCTACTCAT CAGGTGGTAT ACTGGATCAT TCCTTTTGCC ATGGCTTAGA
2110 2120 2130 2140 2150 2160
AACCACTACG TCAAGAAGGA CAGGAAATGG TTCCAGGAAC CATACGCGTA CCCCAAGCAT
2170 2180 2190 2200 2210 2220
CTTGAAACCC ATCCAGAGCT CGCAGATCAA GCATGGCTTT ACAAATCTGT TCTAGAAATT
2230 2240 2250 2260 2270 2280
TGCAGATACT GGGTAGAGCT AAGATATTCC CTCATCCAGC TCCTTTACGA CTGCATGTTC
2290 2300 2310 2320 2330 2340
CAAAACGTGG TCGATGGTAT GCCACTTGCC AGATCTATGC TCTTGACCGA TACTGAGGAT
2350 2360 2370 2380 2390 2400
ACGACCTTCT TCAATGAGAG CCAAAAGTTC CTCGATAACC AATATATGGC TGGTGACGAC
2410 2420 2430 2440 2450 2460
ATCCTTGTAG CACCCATCCT CCACAGCCGT AACGAGGTTC CGGGAGAGAA CAGAGATGTC
2470 2480 2490 2500 2510 2520
TATCTCCCTC TATTCCACAC CTGGTACCCC TCAAACTTGA GACCGTGGGA CGATCAGGGA
2530 2540 2550 2560 2570 2580
GTCGCTTTAG GGAATCCTGT CGAAGGTGGC AGCGTTATCA ACTACACTGC CAGGATTGTT
2590 2600 2610 2620 2630 2640
GCCCCAGAGG ATTATAATCT CTTCCACAAC GTGGTGCCGG TCTACATCAG AGAGGGTGCC
2650 2660 2670 2680 2690 2700
ATCATTCCGC AAATTCAGGT ACGCCAGTGG ATTGGCGAAG GAGGGCCTAA TCCCATCAAG
2710 2720 2730 2740 2750 2760
TTCAATATCT ACCCTGGAAA GGACAAGGAG TATGTGACGT ACCTTGATGA TGGTGTTAGC
2770 2780 2790 2800 2810 2820
CGCGATAGTG CACCAGATGA CCTCCCGCAG TACCGCGAGG CCTATGAGCA AGCGAAGGTC
2830 2840 2850 2860 2870 2880

FIGURE 10 CONTINUED

```
GAAGGCAAAG ACGTCCAGAA GCAACTTGCG GTCATTCAAG GGAATAAGAC TAATGACTTC
      2890       2900       2910       2920       2930       2940
TCCGCCTCCG GGATTGATAA GGAGGCAAAG GGTTATCACC GCAAAGTTTC TATCAAACAG
      2950       2960       2970       2980       2990       3000
GAGTCAAAAG ACAAGACCCG TACTGTCACC ATTGAGCCAA AACACAACGG ATACGACCCC
      3010       3020       3030       3040       3050       3060
TCTAAGGAAG TTGGTAATTA TTATACCATC ATTCTTTGGT ACGCACCGGG CTTTGACGGC
      3070       3080       3090       3100       3110       3120
AGCATCGTCG ATGTGAGCCA GGCGACCGTG AACATCGAGG GCGGGGTGGA ATGCGAAATT
      3130       3140       3150       3160       3170       3180
TTCAAGAACA CCGGCTTGCA TACGGTTGTA GTCAACGTGA AAGAGGTGAT CGGTACCACA
      3190       3200       3210
AAGTCCGTCA AGATCACTTG CACTACCGCT TAG
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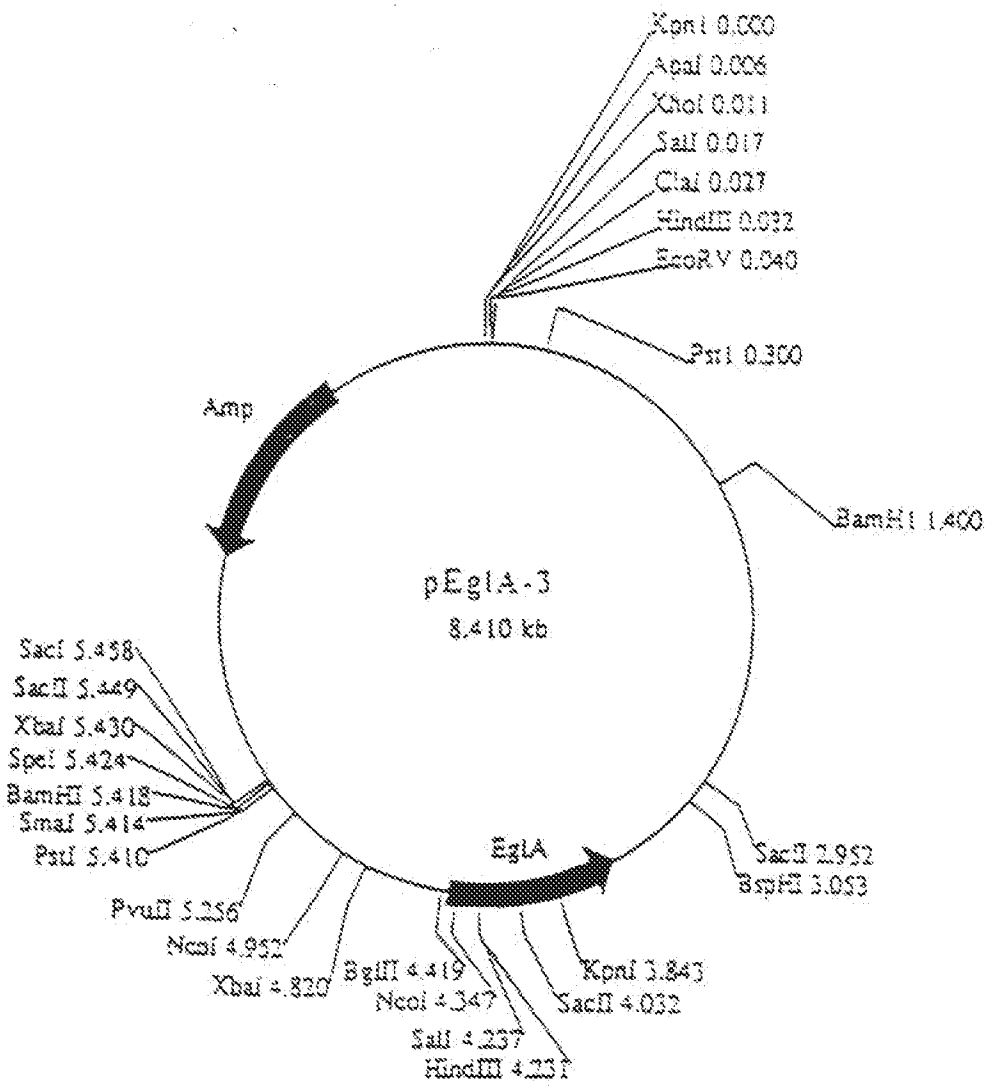
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ApaI 0.006
XhoI 0.011
SalI 0.017
ClaI 0.027
HindIII 0.032
EcoRV 0.040
PstI 0.300
BamHI 1.400
SacII 2.952
BspHI 3.053
KpnI 3.843
SacII 4.032
SalI 4.237
HindIII 4.237
NcoI 4.347
BglII 4.419
XbaI 4.820
NcoI 4.952
PvuII 5.256
PstI 5.410
SmaI 5.414
BamHI 5.418
SpeI 5.424
XbaI 5.430
SacII 5.449
SacI 5.458
pEglA-3
8.410 kb
Amp
EglA

FIG. 11

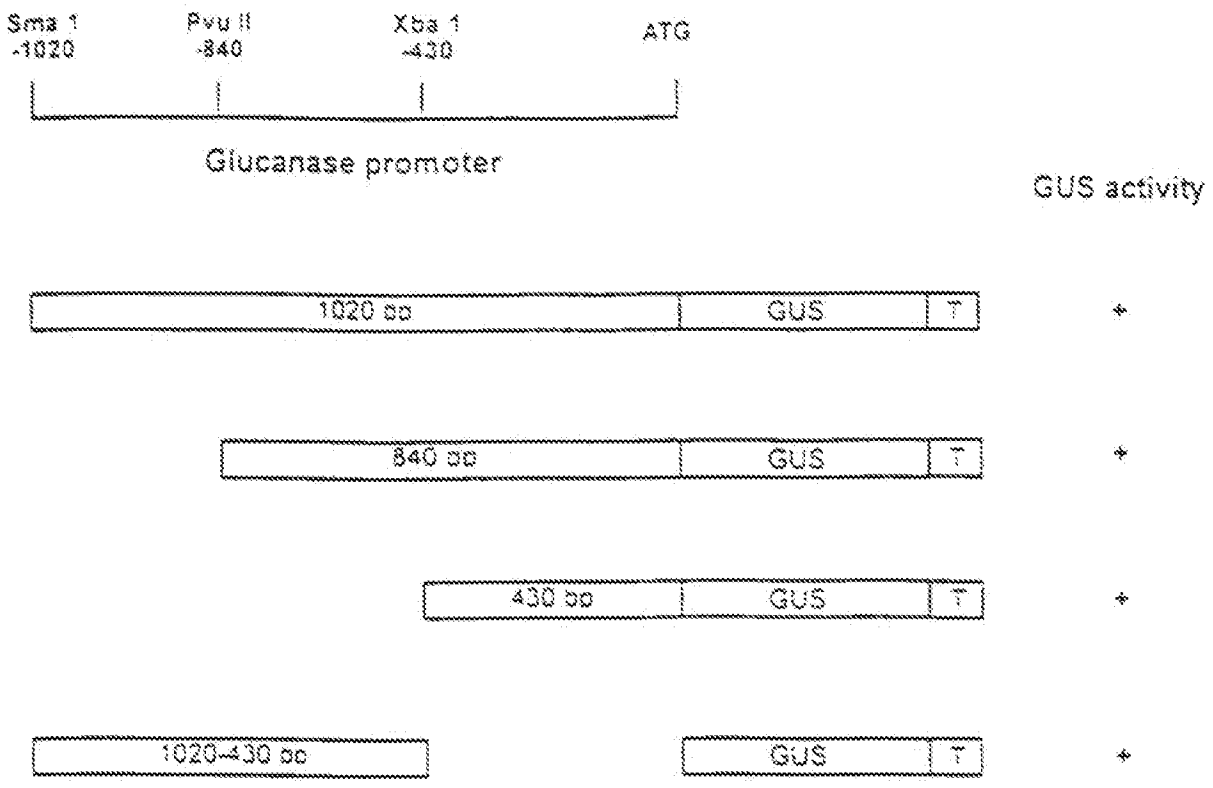
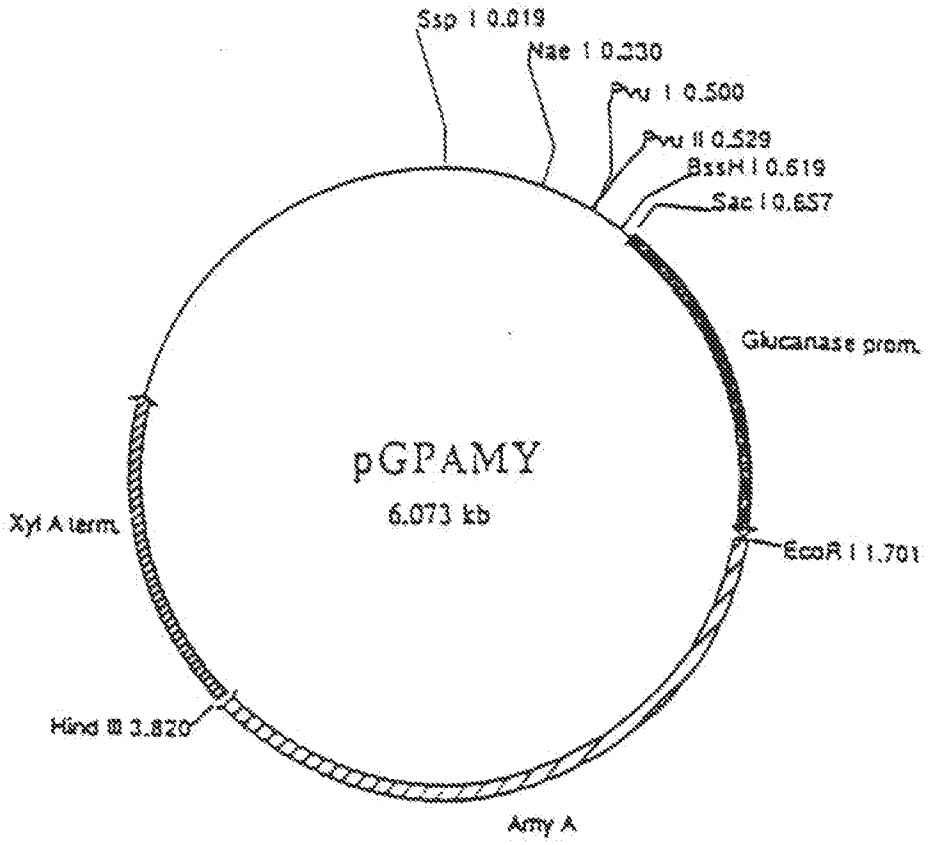

Sma 1
-1020
Pvu II
-840
Xba 1
-430
ATG
Glucanase promoter
GUS activity
1020 bp
GUS
T
+
840 bp
GUS
T
+
430 bp
GUS
T
+
1020-430 bp
GUS
T
+

FIG. 12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Sac I 0.657
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pGPAMY
6.073 kb
EcoR I 1.701
Amy A
Hind III 3.820
Xyl A term.

FIG. 13

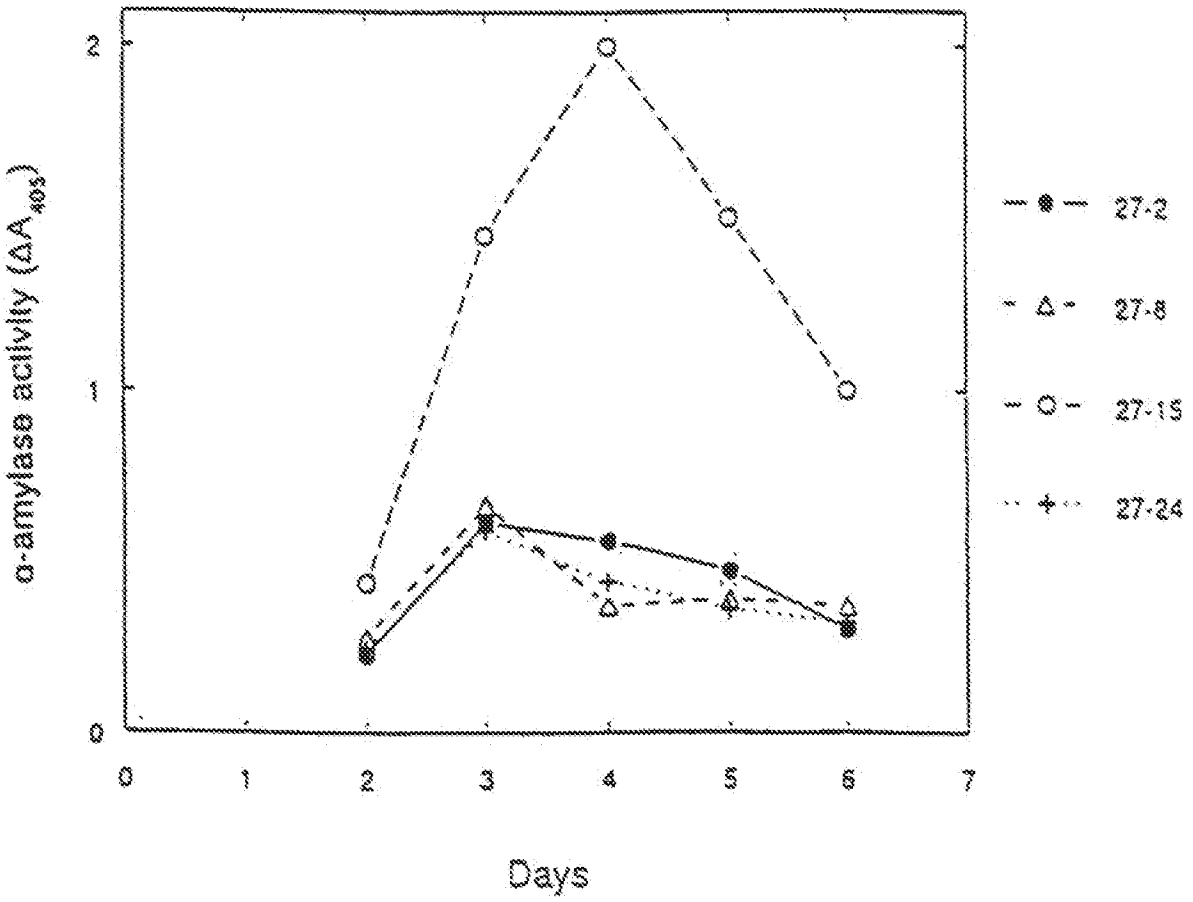
α-amylase activity (ΔA_{405})
2
1
0
0
1
2
3
4
5
6
7
Days
27-2
27-8
27-15
27-24

FIG. 14

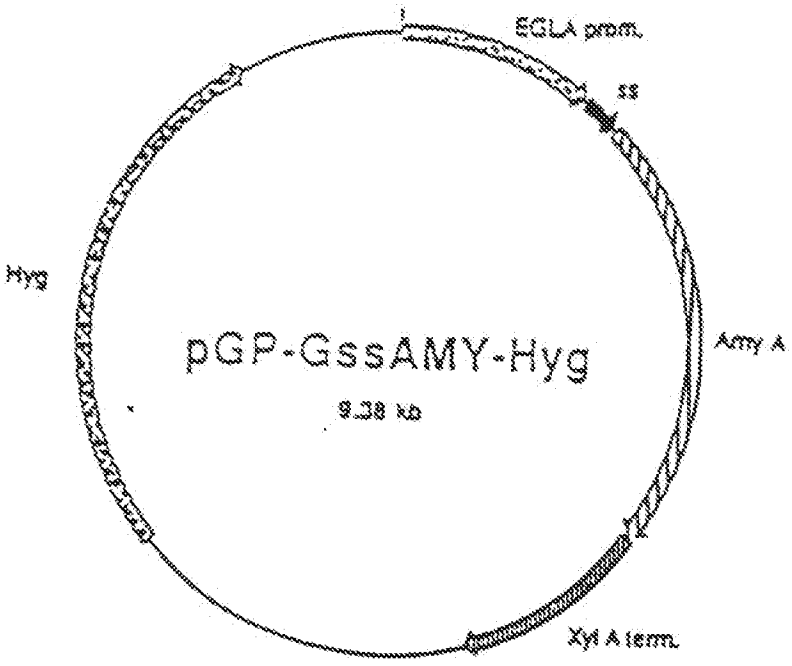

FIG. 15

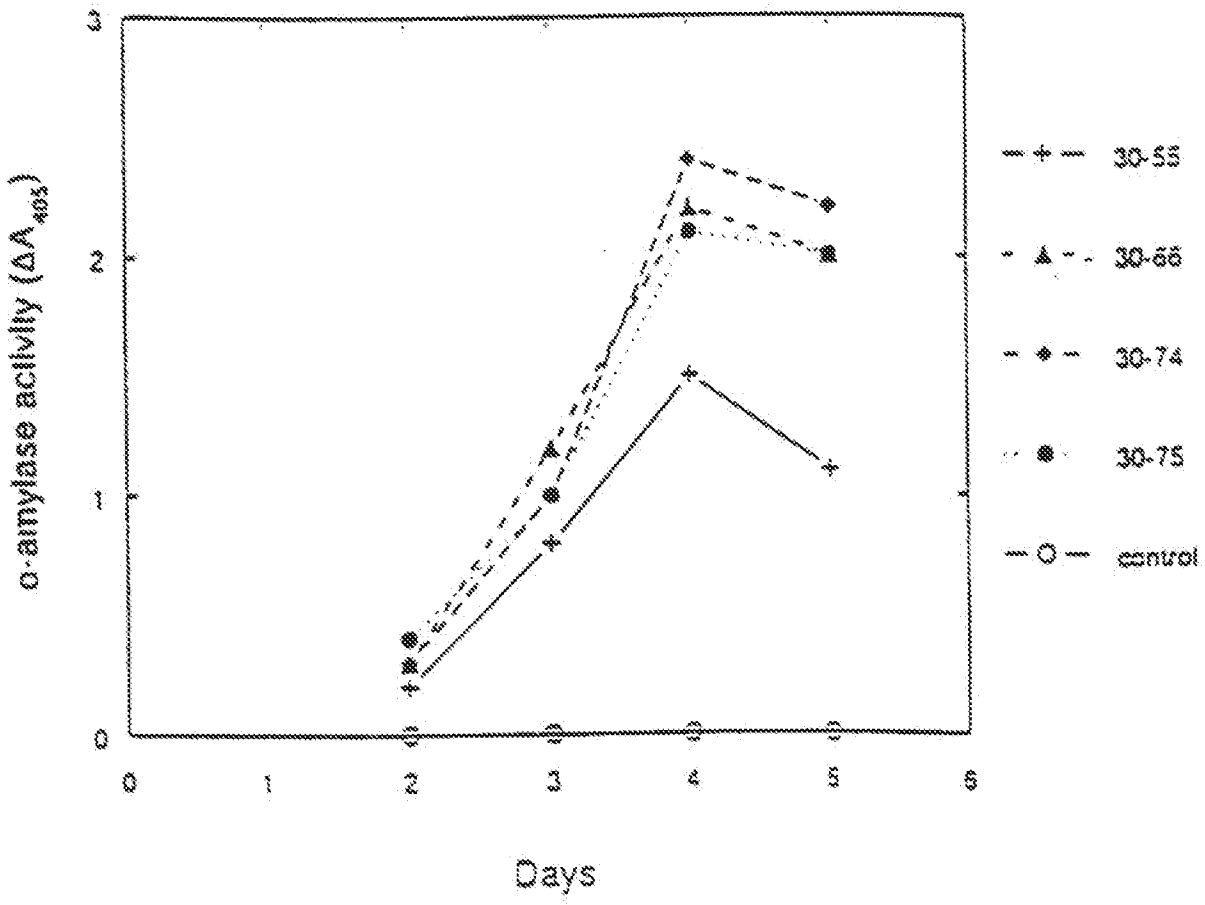
α-amylase activity (ΔA_{405})
0
1
2
3
0
1
2
3
4
5
6
Days
30-55
30-66
30-74
30-75
control

FIG. 16

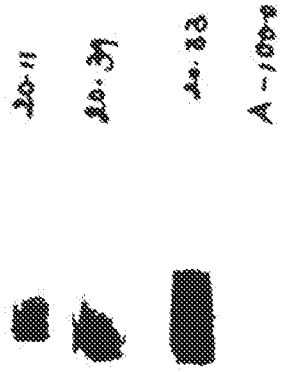

FIG. 17

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 96/01008

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/56 C12N9/42 C12N15/80 C12N15/62 C12N1/15
C12N1/19 C12N5/10 //(C12N1/15,C12R1:66)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL EMFUN:SCD12901;ACCES-NO:D12901 SAKAMOTO,S. ET AL. Cloning and sequencing of the cellulase XP002009466 cDNA from Asprgillus kawachii and its expression in Saccharomyces cerevisiae. 13aug1992; abstr. --- EP,A,0 458 162 (KAO CORPORATION) 27 November 1991 see claims --- -/--	1-9, 13-18, 21-24, 26,28,29 1

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 July 1996	07.08.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Delanghe, L

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. Application No
PCT/EP 96/01008

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NUCLEIC ACIDS RESEARCH, vol. 18, no. 19, 11 October 1990, OXFORD GB, page 5884 XP002009463 TOSHIHIKO OOI ET AL.: "Complete nucleotide sequence of a gene coding for Aspergillus aculeatus cellulase (F1-CMCase)" see the whole document ---	1
Y	AGRICULTURAL AND BIOLOGICAL CHEMISTRY, vol. 49, no. 5, May 1985, TOKYO JP, pages 1257-1265, XP002009464 GENTARO OKADA: "Purification and properties of a cellulase from Aspergillus niger" see the whole document ---	1
P,X	CURRENT GENETICS, vol. 27, no. 5, April 1995, pages 435-439, XP002009465 S.SAKAMOTO ET AL.: "Cloning and sequencing of cellulase cDNA from Aspergillus kawachii and its expression in Saccharomyces cerevisiae" see the whole document -----	1-9, 13-18, 21-24, 26,28,29

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inv ternal Application No
PCT/EP 96/01008

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-458162	27-11-91	JP-A- 4027386	30-01-92
		DE-D- 69116597	07-03-96
		ES-T- 2085375	01-06-96
		US-A- 5258297	02-11-93